

HANDBOOK OF BACTERIOLOGY

For Students and Practitioners of Medicine

by

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DEDICATED

to the Memory of

ALEXANDER CHARLES O'SULLIVAN

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TEACHER, COLLEAGUE, FRIEND

PREFACE TO THE SIXTH EDITION

The ten momentous years which have elapsed since the publication of the fifth edition of this Handbook have, as is inevitable in times of war, witnessed advances in microbiology in various directions and these advances have necessitated many alterations in the text of the book.

The revision in preparation for this, the sixth, edition has been the most complete which the book has received since its publication almost twenty-five years ago. Two chapters have been added and eleven have been completely rewritten. Not one remains without considerable additions, deletions or alterations.

When the last edition was published, the sulphonamides were new discoveries and penicillin, the first of the antibiotics, was of interest only to the bacteriologist. Now two chapters, one new and the other drastically revised, are required to deal with chemotherapy.

Among chapters which have been rewritten are five on immunity, three on intestinal bacteria and one on undulant fever.

That the chapter on Viruses and Rickettsiæ is almost entirely new will not surprise anyone who is familiar with the advances which have been made in the study of these minute organisms, not only in respect of their culture in the embryonated egg and of their morphology as revealed by the electron microscope, but also as regards their pathogenicity and the immunity which they elicit.

The formulæ of various solutions, stains and culture media have been relegated to the last chapter where they will be available for reference without suggesting that the student should attempt to memorise them.

The problems of bacterial nomenclature are still unsolved. In the last edition, some of the new names of genera and species given in Bergey's Manual of Determinative Bacteriology were

used for the first time. In this edition, there has been a further acceptance of the Manual's nomenclature, but I remain unconvinced that we should change a familiar name for an unfamiliar one merely on grounds of priority. So *Staphylococcus* is here used for the genus now called *Micrococcus* in the Manual. The names of the Rickettsiæ given in the Manual have, in general, been accepted, but no attempt has been made to ascribe names, other than those of the diseases they produce, to the pathogenic viruses. In this edition, for the first time, the scientific names of micro-organisms, animals and plants are printed in italics.

The most unwilling student could not regret more than I the increase in the text from 455 to 535 pages. It is much easier to add to a book than to take from it and not even the rigorous pruning which the text has received has permitted the incorporation of new material without this increase in size.

I know that the book is widely used for reference purposes by many who are not technically students and, while I hope that such persons will continue to find it serviceable, I trust that they will realise that some, at least, of the deficiencies which they may note, are due to the fact that it is intended for students rather than for bacteriologists.

It is a pleasure to record my deep indebtedness to my lecturers, Dr. William Hayes and Dr. F. S. Stewart, for all the help they have so cheerfully afforded me in the writing and revision of this edition and for the care with which they have read the proofs. Dr. H. J. Parish was of great assistance to me in the preparation of sections dealing with specific prophylaxis and therapeutics. I am deeply indebted to Dr. C. A. Hoare for the suggestions he was kind enough to make for improvements and corrections in the chapter on pathogenic protozoa.

Many of the illustrations are either new or have been redrawn or rephotographed. I consider myself fortunate in having been able to secure the services of such a well-known medical artist as Mr. Douglas J. Kidd, who is responsible for the coloured plates and for almost all the drawings. Mr. W. Kampff has, once again, acted as photographer. Dr. C. E. van Rooyen kindly gave per-

mission for the reproduction of fig. 89. For the inclusion of the six electron micrographs shown in figs. 2, 49, 75, 91, 93 and 94, I am indebted to the authors of the papers in which they originally appeared (Messrs. T. F. Anderson, M. Delbrück, R. H. Green, F. Heinmets, S. E. Luria, H. E. Morton, S. Mudd, H. Plotz and J. E. Smadel), and to the editors and proprietors of the journals (*Journal of the American Medical Association*, *Journal of Bacteriology* (Williams and Wilkins Company) and *Journal of Experimental Medicine*) in which their papers were published.

Finally, I trust that this, the sixth, edition will be not less appreciated by students of bacteriology, for whom it was written, and by their teachers than were the first five editions.

JOSEPH W. BIGGER.

DEPARTMENT OF BACTERIOLOGY,
TRINITY COLLEGE, DUBLIN,
September, 1949

PREFACE TO THE FIRST EDITION

The publication of a new Handbook may seem to demand, if not an apology, at least an explanation, in view of the large number of such works available.

This volume is the outcome of the dissatisfaction with the existing text-books, frequently expressed by students to the author. The complaint is that the text-books contain seven to eight hundred pages, and the student has not sufficient time to master their contents in the few months devoted to the study of Bacteriology in the medical curriculum, and further that he is unable to distinguish the more important matter from the less. The result often is that the student relies, for examination purposes, on grinders' notes or "cram" books. I feel that this criticism must be taken seriously, since the majority of text-books attempt both to supply the necessary information to the student, and also

PREFACE TO THE FIRST EDITION

to the laboratory reference books. This criticism is not made in the spirit of antagonism to those volumes to which I have been deeply in debt in preparing this book, and which are constantly on the bench in my own laboratory. I have, however, felt for some years the urgent need of a shorter text-book, suitable for the use of students, and, as no such work appeared, I set myself the task of writing one. Whether I have succeeded or not will be for my readers, particularly my student readers, to say, for it is their need that I have ever had in mind.

This work is primarily intended for students of Medicine, and for those practitioners who have not specialized in Bacteriology. When its contents have been assimilated, I would recommend any one wishing to learn more of the subject to consult one of the larger text-books. I believe that here the student will find all the more important facts relating to bacteria as they affect man. I have endeavoured, as far as possible, to present the practical aspect of the subject, to reduce the theoretical, and to keep prominently before the reader the fact that bacteria are important to the physician, not in themselves, but only as the causes of disease. Some criticism may be directed to a certain dogmatism, in the presentation, but I believe that it is better to be dogmatic, putting forward a probable theory as the correct one, rather than to leave the student confused with the claims of half a dozen rival theories, concerning the relative merits of which only the advanced worker is in a position to decide.

JOSEPH W. BIGGER.

SCHOOL OF PATHOLOGY,
TRINITY COLLEGE, DUBLIN,
December, 1924.

CONTENTS

<i>Chapter</i>		<i>Page</i>
I	INTRODUCTION	1
II	MICROSCOPIC EXAMINATION OF BACTERIA	15
III	STERILISATION	37
IV	THE PREPARATION AND USE OF CULTURE MEDIA	43
V	THE MAKING OF CULTURES	63
✓VI	SPECIAL TECHNIQUE	82
✓VII	SEROLOGICAL TECHNIQUE	102
<u>VIII</u>	THE OBTAINING OF MATERIAL FROM THE PATIENT FOR BACTERIOLOGICAL EXAMINATION	125
✓IX	THE IDENTIFICATION OF PATHOGENIC BACTERIA	137
<u>X</u>	ANTISEPTICS, CHEMOTHERAPY, ANTIBIOTICS	149
<u>XI</u>	PRACTICAL METHODS IN CONNECTION WITH ANTIBIOTICS	165
✓XII	BACTERIOLOGY OF WATER, MILK AND SHELLFISH	175
XIII	BACTERIA IN HEALTH AND DISEASE	185
✓XIV	INTRODUCTION TO IMMUNITY	199
✓XV	ANTIGENS AND ANTIBODIES	208
✓XVI	PHAGOCYTOSIS	215
✓XVII	TOXINS AND ANTITOXINS	219
✓XVIII	PRECIPITATION AND AGGLUTINATION	226
✓XIX	THE KILLING AND DISSOLVING OF BACTERIA AND OTHER CELLS	238
✓XX	HYPERSENSITIVITY	243
✓XXI	THE CLASSIFICATION OF BACTERIA	250
✓XXII	STAPHYLOCOCCI	256
✓XXIII	STREPTOCOCCI	263

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ILLUSTRATIONS

COLOURED PLATES

<i>Plate</i>	<i>Facing Page</i>
I. Bacteria as seen with the Oil-Immersion Lens	32
II Colonies of Bacteria	144
III. C. Diphtheriæ	304
IV Malaria Parasites in Blood Films	496

FIGURES

<i>Figure</i>	<i>Page</i>
1 Flagella, capsules and spores	1
2 Bacilli with flagella	5
3 Types of cocci	11
4 Bacilli, vibrios, spirilla and spirochætes	11
5 Hanging drop preparation using hollow ground slide	19
6 Hanging drop preparation using ordinary slide and ring	19
7 Method of spreading blood film	21
8 Hot air steriliser	38
9 Steam steriliser (Arnold pattern)	39
10 Autoclave	40
11 Sterile graduated pipette	42
12 Three Durham tubes	52
13 Angle centrifuge	64
14 Platinum wire	64
15 The preparation of a Pasteur pipette	64
16 Spreader	66
17 The method used to inoculate a tube of medium	69
18 Pouring a plate	69
19 Spreading a plate by method of parallel streaks	72
20 The colonies on a plate spread by the method of parallel streaks	73
21 Colonies on a plate inoculated by spreading	74
22 Agar slope in tube	76
23 Agar slope in screw cap bottle	76
24 The McIntosh and Fildes electric anaerobic jar	78
25 Small Chamberland filter	87
26 Berkefeld filter	88

Chapter	Page
XXIV <i>Diplococcus pneumoniae</i> ✓	276
XXV <i>Neisseria meningitidis</i> ✓	283
XXVI <i>Neisseria gonorrhoea</i> ✓	288
XXVII <i>Bacillus anthracis</i>	293
XXVIII <i>Corynebacterium diphtheriae</i>	302
XXIX COLIFORM BACTERIA	317
XXX SALMONELLÆ	325
XXXI ENTERIC FEVER	335
XXXII BACILLARY DYSENTERY ✓	347
XXXIII MISCELLANEOUS BACTERIA ✓	354
XXXIV <i>Vibrio cholerae</i>	360
✓ XXXV <i>Posteurella</i>	365
XXXVI HAEMOPHILIC BACTERIA	374
XXXVII BACTERIA CAUSING UNDULANT FEVER	380
✓ XXXVIII THE TUBERCLE BACILLUS	384
XXXIX <i>Mycobacterium leprae</i>	402
✓ XL <i>Malleomyces mallei</i>	405
✓ XLI SPIROCHAETES	408
XLII TOXIN PRODUCING CLOSTRIDIA	424
XLIII ANAEROBIC BACTERIA OF WOUNDS	435
XLIV ACTINOMYCOSIS	447
XLV VIRUSES AND RICKETTSIÆ	452
XLVI PATHOGENIC PROTOZOA	491
XLVII PATHOGENIC FUNGI	511
XLVIII FORMULÆ	519
INDEX	537

ILLUSTRATIONS

xv

Figure	Page
69 Tubercle bacilli in section of lung	388
70 Tubercle bacilli in deposit from urine	391
71 <i>Mycobacterium leprae</i> in film from nose	403
72 <i>Borrelia obermeyerii</i> in blood	409
73 <i>Treponema pallidum</i> from tonsil	412
74 <i>Treponema pallidum</i> in lymph gland	413
75 <i>Leptospira icterohæmorrhagica</i>	417
76 <i>Leptospira icterohæmorrhagica</i> in section of kidney of infected guinea-pig	418
77 Spirochetes and fusiform bacilli from throat of a case of Vincent's angina	421
78 Spirochetes and other bacteria from tooth	422
79 <i>Cl. tetani</i> from agar culture	425
80 <i>Cl. perfringens</i> from agar culture	436
81 Stormy fermentation of milk by <i>Cl. perfringens</i>	437
82 <i>Cl. perfringens</i> in muscle	438
83 <i>Cl. septicum</i> from broth culture	440
84 <i>Cl. sporogenes</i> from broth culture	443
85 <i>Actinomyces bovis</i>	448
86 Colony of <i>Actinomyces bovis</i> in liver	449
87 Streptothrix in pus	450
88 Elementary bodies of a virus	453
89 Viruses and bacteria	460
90 Negri body in nerve cell	462
91 Vaccinia virus	473
92 Yellow fever inclusion body	475
93 <i>Bact. coli</i> with adsorbed phage particles	486
94 <i>R. mooseri</i> in endothelial cell of guinea-pig testis	489
95 Diagram of the development of the malaria parasite	494
96 Macrogametocyte of <i>P. falciparum</i>	495
97 Entamoebæ	500
98 Diagram of trypanosome	502
99 <i>Trypanosoma evansi</i> in rat blood	503
100 <i>Leishmania donovani</i> in section of spleen	507
101 <i>Leishmania tropica</i> in smear from oriental sore	509
102 <i>Microsporon andouinii</i> in hair	512
103 Colony of <i>Microsporon andouinii</i> on glucose agar	513
104 <i>Trichophyton endothrix</i> in hair	514
105 <i>Trichophyton megalosporon</i> in scale of skin	514
106 Colonies of <i>Trichophyton</i> on glucose agar	515
107 Colonies of <i>Achorion schoenleini</i> on glucose agar	515
108 Colonies of <i>Sporotrichum beurmanni</i>	517
109 <i>Momilia</i> from broth culture	517

Figure

Figure	Page
27 Seitz filter	88
28 Action of phage on <i>Salmt. typhi</i>	99
29 Slide agglutination	103
30 Microscopic agglutination	104
31 Apparatus for agglutination test (Dreyer's method)	106
32 Macroscopic agglutination test (Dreyer's method)	107
33 Deposited bacteria (Felix's method)	109
34 Sterile swab	126
35 All-glass syringe in large test tube	132
36 Blood culture bottle	133
37 Behring venule	135
38 Variant colonies of <i>Staphylococcus pyogenes</i>	141
39 Uniform turbidity of <i>Salmt. typhi</i> and deposit of streptococci in broth	142
40 Liquefaction of gelatin by <i>Staph. pyogenes</i>	143
41 Test of sensitivity to penicillin by half plate method	166
42 Test of sensitivity to penicillin by gutter plate method	167
43 Assay of penicillin by cup plate method	169
44 Phagocytosis of streptococci by polymorphonuclear leucocyte	216
45 <i>Staphylococcus pyogenes</i> from agar culture	257
46 <i>Streptococcus pyogenes</i> from broth culture	266
47 <i>Streptococcus pyogenes</i> in pus	269
48 Pneumococci in film of pus stained to show capsules	278
49 Pneumococci showing Neufeld reaction	282
50 Meningococci in purulent cerebro-spinal fluid	284
51 Gonococci in pus	286
52 Edge of colony of <i>B. anthracis</i> on agar	29
53 <i>B. anthracis</i> in gelatin	294
54 <i>B. anthracis</i> from agar culture	295
55 <i>B. anthracis</i> in section of kidney of infected guinea-pig	298
56 <i>C. diphtheriae</i> from serum culture	302
57 <i>C. diphtheriae</i> in false membrane	303
58 <i>C. pseudodiphtheriticum</i> from culture on serum	315
59 <i>Bact. coli</i> in deposit of urine from case of cystitis	320
60 <i>Bact. pneumoniae</i> showing capsules	323
61 <i>Salmt. typhi</i> from agar culture	326
62 <i>L. acidophilus</i> from milk culture	358
63 <i>V. cholerae</i> from agar culture	360
64 <i>Past. pestis</i> from young agar culture	366
65 <i>Past. pestis</i> in fluid from bubo	368
66 <i>Myco. tuberculosis</i> from culture	385
67 Culture of tubercle bacillus (human variety)	386
68 Culture of tubercle bacillus (human variety) on surface of glycerol broth	387

CHAPTER I

INTRODUCTION

Bacteria are minute, rigid, unicellular masses of protoplasm of apparently very simple structure. Their position in the scale of living organisms cannot be regarded as exactly determined, since they possess many characteristics similar to those of both the simpler plants and animals. They may be considered as forming a more primitive type than either of these, intermediate between the yeasts and moulds on the one hand and the protozoa on the other. They differ from the algae, which in many respects they

disease in man and other animals. It may be said, however, that the popular conception of bacteria as harmful and unnecessary parasites is very far from the truth. It is almost certain that human life on the earth would be impossible were it not for the innumerable activities of bacteria. They form the link between the animal and vegetable kingdoms and render the dead and useless material of the former available for the growth of plants, on which the life of all animals depends. Further, they fix atmospheric nitrogen and render it available for plant life, which explains the fact that the soil of the earth, suitably tended, is always capable of sustaining vegetation. They are the active agents in many phenomena which are taken so much for granted

depend on the action of bacteria.

The size of bacteria is somewhat varied, but one may say that the

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artefacts produced by the rotatory movements of the bacteria rather than the agents responsible for motility. The motile organisms are chiefly bacilli, vibrios and spirochaetes: few of the cocci possess this characteristic. Some bacteria are provided with flagella all around their bodies (peritrichous), others with tufts at one or both ends, and yet others with a single flagellum at each end, or at one pole only. A bacterium provided with flagella is almost certainly motile, even if no movement can be observed

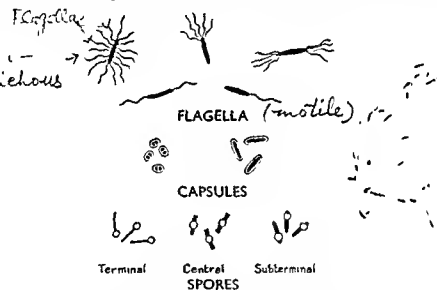


FIG 1—FLAGELLA, CAPSULES AND SPORES

at a given time, and the absence of flagella is, except in a few species, indicative of the organism being non-motile. In examining for motility it is necessary to distinguish true motility from the vibratory movement shown by minute particles, suspended in fluids, which is known as Brownian movement.

Bacteria multiply, very simply, by fission. They are, so far as is known, entirely asexual. When mature, if there is a sufficiency of food material available and if other conditions are satisfactory, the individual increases somewhat in size and a constriction appears: this becomes more marked, and finally a septum is pro-

average is about 1 micron in diameter. A micron (μ) is 1/1000 millimetre or 1/25,000 inch. It is obvious, therefore, that in order to be able to see them, a microscope affording a considerable degree of magnification is essential.

When examined in the living condition bacteria are seen to be transparent, colourless, and homogeneous or finely granular. Their presence in liquid, when examined microscopically, can be detected only on account of their refractivity. When killed and suitably stained they are much more easily seen, but very little more information as to their structure is to be made out. Until comparatively recently no nucleus had been satisfactorily demonstrated in bacteria and it was generally believed that there was no complete differentiation of cytoplasm and nucleoplasm. Evidence has now accumulated, however, that, by the use of suitable methods, a discrete nuclear apparatus can be demonstrated in bacteria of various species. There is certainly no doubt that chemical substances characteristic of nuclear material occur in bacteria.

Bacteria are fairly definite in shape, owing to the existence of a cell envelope. This envelope is probably only a specialised part of the ectoplasm and is rarely, if ever, composed of cellulose. In some, the envelope is surrounded by a soft jelly-like material, the capsule, which may have a width greater than the diameter of the bacterium which it surrounds. The presence of capsules often causes a mass of bacteria to adhere together and to behave somewhat like mucus when touched with a wire.

All bacteria require for their growth a considerable amount of water, and they usually flourish, most abundantly, in a moist medium. When observed in the living state, in fluid, some are found to move freely, others to remain motionless. The motility of bacteria is due, in some types, to bending of the whole body of the organism and, in others, to rotation around its long axis. In general, however, motility, when it occurs, is attributed to the action of very thin, but relatively long, contractile, hair-like processes, the flagella, with which most motile bacteria are provided. Some workers, and notably Pijper, consider that the flagella are

hydrogen and oxygen, bacteria contain phosphorus together with small amounts of sulphur, calcium, sodium, potassium, magnesium, iron, and other elements.

During the growth of bacteria there occurs an active synthesis of the complex constituents of the cell. In order that this synthesis may take place, the elements, which are ultimately incorporated in the cell protoplasm, must be present in an assimilable form in the medium in which the organism is growing. Phosphorus,



FIG 2 —BACILLI WITH FLAGELLA (Electron microscope).

sulphur, sodium, potassium, iron and other elements required in small amounts are readily assimilated from simple salts present in the environment of the organism. Different types of bacteria, however, show considerable differences in the nature of the food material which they can accept as sources of the elements carbon and nitrogen. These differences correspond in general with differences in the synthetic power of the bacteria. Some organisms (autotrophs) are endowed with considerable synthetic power and

duced, which divides the original cell into two individuals, each of which rapidly grows to adult size. The new individuals may separate almost longer or shorter envelopes. Uncapable of becoming from 20 minutes.

accumulation of harmful waste products and to other factors, this rate of reproduction is not long maintained. Even if fission occurred only once each hour, the number of bacteria produced from a single individual, in 24 hours, would be almost 17 millions.

Certain bacteria, chiefly bacilli, are able to protect themselves against adverse circumstances by the formation of spores. This is not a means of reproduction, since an individual produces not more than one spore and, in doing so, dies. The object is the continuance of the race in conditions which would destroy all the vegetative forms of the bacteria. The spore in a bacillus may be situated at, or near, the middle (central), at one end (terminal), or near an end (sub-terminal). Usually a bright, highly refractile spot is first seen; this increases in size until it becomes a round or oval body, shorter than the long axis of the bacillus, but often of greater diameter than the width of the bacillus, in which it produces a bulge. When the spore is fully developed, the body of the bacillus disappears and the spore, which is non-motile, is free. The spore possesses a relatively thick and very resistant membrane, its vital functions are reduced to a minimum and it is capable of surviving the absence of food and water for long periods and exposure to a temperature which would be sufficient to kill the vegetative form of the bacillus in a short time. When external conditions are again favourable, the spore-membrane ruptures and the bacillus itself is reproduced.

The chemical composition of bacteria varies in different species. All, however, consist chiefly of water, which forms from 70 to 85 per cent of their weight. Of the rest, the greater part is some form of protein material together with carbohydrates, waxes, fats, lipoids and mineral matter. In addition to carbon, nitrogen,

INTRODUCTION

to a suitable hydrogen acceptor from which in turn it may be passed on to further hydrogen acceptors.

Two main types of oxidation may be distinguished first, that occurring under aerobic conditions and associated with the

as a result of dehydrogenase action. They differ, however, in that in fermentation the final hydrogen acceptor is not oxygen but some intermediate metabolic product. Generally, respiration is a more efficient method of metabolism than fermentation since it results in more complete oxidation, thus liberating a greater amount of energy.

The fermentation of glucose by a number of bacterial species has been shown to be similar in its early stages to the Embden-Meyerhof-Parnas scheme for muscle and yeast involving oxidation, phosphorylation and splitting of the glucose molecule. From pyruvic acid, a key intermediary in this scheme, a variety of compounds may be produced by different bacteria—formic, acetic, propionic, lactic, butyric and succinic acids; ethyl, propyl and butyl alcohols; acetylmethyl carbinol and 2 : 3 butylene glycol. Some organisms can break down the formic acid produced in glucose fermentation to hydrogen and carbon dioxide. Although fermentation is essentially an anaerobic process, aerobic oxidation of phases of the typical ferment with anaerobic bic conditions

In almost all aerobic oxidations, respiration is dependent on the respiratory pigment cytochrome. Cytochrome is a haematin protein capable of alternate oxidation and reduction and functions as a carrier of hydrogen from substrate to oxygen. Some dehydro-

can utilise very simple inorganic compounds, such as carbon dioxide and nitrates or ammonium salts, as sources of carbon and nitrogen respectively. From these they can build up their complex carbohydrates, proteins, fats and other essential constituents.

The bacteria in which we are mainly interested are classed as heterotrophs and, as such, have much less synthetic power than the autotrophs and, in consequence, require much more complicated organic food materials. It is, indeed, possible to trace a gradual loss of synthetic power, reflected in increasing complexity of nutritional requirement, as we pass from the less to the more highly parasitic types. It is probable that this loss is primarily the result of the acquisition of the parasitic habit which ensures for these organisms an abundant supply of preformed food materials in the body of the host.

All the pathogenic bacteria require an organic source of carbon such as is found in carbohydrates, proteins, amino acids and fatty acids: these compounds also supply hydrogen and oxygen. In respect of their nitrogen requirements, however, they show considerable differences. Some, such as *Bact. coli*, which are, in the main, saprophytes, can obtain the nitrogen they require from ammonium salts. The more highly pathogenic bacteria, however, require the presence in the medium of one or more preformed amino acids. These amino acids are usually supplied in artificial media in the form of peptones and polypeptides. Complex proteins having larger molecules cannot be directly assimilated by the cell but must first be broken down into smaller units. It is, therefore, only bacteria capable of producing proteolytic enzymes which can utilise them.

Energy, which is required mainly for synthesis, is most readily obtained by the oxidative breakdown of carbohydrates of which the one present in largest amount in the body fluids is glucose.

Biological oxidation consists, not in the addition of oxygen to the substrate, but in the removal of hydrogen from it. This removal of hydrogen is catalysed by enzymes known as dehydrogenases. The hydrogen removed is not liberated in the gaseous state but is transferred through the agency of the dehydrogenase

tion of pure bacterial toxins where it is desirable to reduce unwanted material to a minimum. They have little, if any, application in routine bacteriological work.

Bacteria may also show specific characteristics in relation to their gas requirements.

only in the presence of anaerobes, grow only when all traces of oxygen are absent from the medium. aerobic bacteria, however, are inhibited by the presence of oxygen. It has been understood that this is due to the production of hydrogen peroxide, a substance highly toxic to bacteria, coupled with a lack of the detoxicating mechanism—catalase—of aerobic organisms. An alternative possibility is that the obligatory anaerobes possess enzymes which, for activity, must be in the reduced state. Most, if not all, bacteria appear to require the presence of carbon dioxide in small amounts and some, such as *B. abortus*, in fact

in some marine species to 70 in the thermophilic bacteria of decaying manure and of hot springs. For each type there is a minimum, an optimum and a maximum temperature of growth. In the case of many pathogenic micro-organisms, the minimum is from 15° to 20°, the optimum about 37°, and the maximum 42° to 43°. Although growth ceases at temperatures below the minimum and above the maximum, death does not necessarily occur, even with prolonged exposures to these temperatures. Extreme cold is especially well borne, and many bacteria which are pathogenic for man are capable of surviving an exposure to

In addition to building material and energy supplying substances, bacteria require what are known as "essential growth factors". These are analogous to and, indeed, in some cases are identical with the animal vitamins. They may be defined as organic compounds essential for the growth of bacteria and active in very low concentrations. The more important growth factors that have been described fall into three classes—(a) the B group of vitamins: biotin, nicotinic acid, pantothenic acid, pyridoxin, para-aminobenzoic acid, riboflavin, thiamine, inositol and folic acid; (b) miscellaneous substances of known composition such as purines, pyrimidines, β -alanine, pimelic acid, haematin and glutamine; (c) substances of unknown composition.

It is probable that most bacteria require all or practically all these factors and that they can synthesise the majority of those they require from simpler substances. Those they cannot synthesise must be supplied preformed in the medium. The power of bacteria to synthesise growth factors is of great importance in connection with animal nutrition since animals can utilise vitamins produced by bacteria in the intestine.

Some of these growth factors such as thiamine, riboflavin, nicotinic acid, pyridoxin and haematin have been shown to be constituent parts of co-enzymes. It is probable, though this has not been proved, that others have similar functions. As will be seen in a later chapter, some important antibacterial substances appear to act by interfering with the function of essential growth factors.

Bacteria in the body have an ample supply of their required growth materials. In the laboratory, however, it is essential, when attempting to grow a particular organism, to ensure that the medium satisfies all its nutritional requirements. Blood or other body fluids can usually be relied on to supply a considerable variety of food materials such as amino acids and growth factors lacking in the simpler laboratory media. When the needs of an organism are exactly known, it is possible to prepare a synthetic medium satisfying these and containing only known constituents. Such defined media have been particularly valuable in the produc-

Every coccus, when fission occurs, is for a time a diplococcus. In some, however, the stage is only temporary while, in the true diplococci, it is more or less permanent. A time comes when each of a pair is about to divide, and then, usually, the two part and each becomes a pair of diplococci. In the streptococci, after fission, the individuals do not readily part and since, in these,

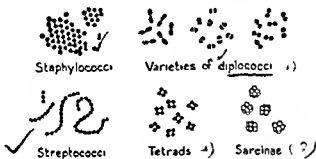


FIG 3—TYPES OF COCCI

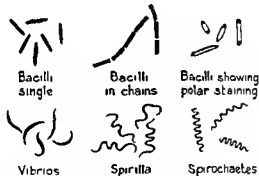


FIG 4—BACILLI, VIBRIOS, SPIRILLA AND SPIROCHAETES

fission proceeds in only one plane, a chain of cocci is produced. If fission proceeds in two planes, producing groups of four, and then of eight, resembling corded bales. If fission proceeds in three planes, producing groups of eight, resembling corded bales.

The bacilli are cylindrical bacteria which may be either straight

resistance is greatly increased and even such a delicate organism as the pneumococcus, when dried over calcium chloride, may survive exposure to a temperature of 115° for half an hour. Spores are very much more resistant and may not be killed, when moist, at the temperature of boiling water for ten or more minutes: in the dry state, some can withstand several hours' exposure to a temperature of 130° to 140° . In speaking of the thermal death point of bacteria it is essential to specify not only the temperature and the time of exposure, but also the material in which suspended.

Ultra-violet rays are most injurious to bacteria; many species are very quickly killed by these agencies, and even some highly resistant spores are destroyed by sunlight in a few hours. Ultra-sonic waves rapidly kill bacteria suspended in fluids.

Bacteria derive their name from the fact that the earliest types examined were rod shaped. The term "bacterium" is now, however, applied to all these micro-organisms and bears no relation to their morphology. Five morphological classes of the lower bacteria are recognised:

1. Cocci.
2. Bacilli.
3. Vibrios.
4. Spirilla.
5. Spirochaetes.

Cocci are more or less spherical organisms. Among them the perfect sphere is uncommon and an oval bacterium is generally included in this group provided its greater axis is not more than double the length of its lesser. Their behaviour after fission enables them to be divided into sub-groups:

- (a) Diplococci.
- (b) Streptococci.
- (c) Staphylococci.
- (d) Tetrads.
- (e) Sarcinae.

Lastly, we have what is probably a large and heterogeneous group of living organisms which, until we can learn more about them, may be grouped together as viruses. These organisms are of such a minute size that they are either invisible or only barely

Safety in the Laboratory

The following chapters describe the practical methods used in the study of bacteria but, before proceeding to consider these, it is essential to warn the student of the dangerous nature of the materials with which he will work in the bacteriological laboratory and to point out to him the precautions which he should take both in his own interests and in the interests of others

The bench should be covered with some impervious substance. If any material containing bacteria is spilled the bench should be im

other pieces of apparatus which have come in contact with bacteria.

The worker should have on his bench a jar containing 2 per cent. lysol to receive slides, pipettes and other small contaminated articles. Plates and test tubes may be temporarily disposed of by immersion in a pail containing 2 per cent. lysol, care being taken, by removing the covers of plates and the plugs of tubes, to ensure the admission of the fluid to their interiors. A few hours' immersion in this disinfectant will kill the majority of pathogenic bacteria but, when material containing dangerous sporing organisms is dealt with, the whole pail should be autoclaved. Alternatively, treatment with a disinfectant may be dispensed with, contami-

long axis is at least twice right angles to their long according to their mode of division, would be into those which occur singly, diplobacilli and streptobacilli. These terms are sometimes used, but are not generally employed to distinguish different types. The bacilli are divided into a number of genera which will be dealt with later.

The rigid curved rods, the curve of which forms less than one complete spiral, are called vibrios. Each is usually provided with a single terminal flagellum.

Longer, rigid, curved organisms, usually with several spirals and with tufts of flagella at one or both poles, are spirilla.

Spirochaetes, the next group, are believed by some to be more correctly classed as protozoa than as bacteria. In them, the number of spirilla, the organisms

the bacilli and the spirilla are met with, and it is occasionally very difficult to say in which group a given organism should be put. In deciding the question regard should be paid only to the or on suitable conditions are

forms. It must also be recognised that certain species are highly

a coccus and, under others, may occur in long filaments.

The members of the next group to be considered differ in many ways from the preceding, and morphologically appear more nearly related to the fungi. They are characterised by the formation of filaments which may exhibit true branching. Branching is not an exclusive property of these, the so-called "higher bacteria", as under certain conditions branching has been observed among such representatives of the lower bacteria as the tubercle bacillus and *C. diphtheria*.

Lastly, we have what is probably a large and heterogeneous group of living organisms which, until we can learn more about them, may be grouped together as viruses. These organisms are of such a minute size that they are either invisible or only barely visible with the highest magnifications of the optical microscope.

Safety In the Laboratory

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or slightly curved, long or short. Their long axis is at least twice their diameter. They always divide at right angles to their long axis, so the only possible classification, according to their mode of division, would be into those which occur singly, diplobacilli and streptobacilli. These terms are sometimes used, but are not generally employed to distinguish different types. The bacilli are divided into a number of genera which will be dealt with later.

The rigid curved rods, the curve of which forms less than one complete spiral, are called vibrios. Each is usually provided with a single terminal flagellum.

number of spirals is usually greater than in the spirilla, the organisms are flexible and do not possess flagella. x

Forms intermediate between the cocci and bacilli and between the bacilli and the spirilla are met with, and it is occasionally very difficult to say in which group a given organism should be put. In deciding the question regard should be paid only to the morphology of the bacterium when growing in or on suitable medium, for many micro-organisms, if the cultural conditions are unsuitable, appear abnormal in shape, the so-called 'involution forms'. It must also be recognised that certain species are highly

a coccus and, under others, may occur in long filaments.

The members of the next group to be considered differ in many respects from the preceding and are characterized by the fact that under certain conditions branching has been observed among such representatives of the lower bacteria as the tubercle bacillus and *C. diphtheriae*.

CHAPTER II

MICROSCOPIC EXAMINATION OF BACTERIA

On account of the extremely small size of bacteria, a microscope is essential for their study. To secure sufficient magnification a $\frac{1}{12}$ -inch objective is a necessity and, in addition to this, we require a lens of somewhat lower power, about $\frac{1}{6}$ of an inch. The $\frac{1}{12}$ -inch lens is an "oil-immersion" lens since, in using it, a drop of cedar oil is placed on the object to be examined and the lens is immersed in the oil. A modern substitute for cedar oil is "polyric oil", made by mixing a liquid synthetic resin, polymethylstyrene, and castor oil. It is cheap to prepare and has the advantage of remaining fluid for long periods on slide or objective, unlike cedar oil which dries and hardens. An oil-immersion lens gives better illumination than a dry lens of the same power, for, when rays of light pass from object to lens through air, some are lost by refraction and others by reflection, since cedar oil has practically the same refractive index as glass, neither of these losses occurs with an oil-immersion lens. Such a lens also permits of a slightly greater working distance between the object and the lens and possesses the further advantage that greater detail can be made out than would be possible with a dry lens. Where cover slips are used with an oil-immersion lens they must be very thin (No. 1).

Further essentials in a microscope are a plane and concave mirror, a sub-stage condenser, which concentrates the light on

incandescent gas-burner. Artificial light is usually to be preferred to daylight. A gas-filled, metal-filament electric bulb is probably the best source of illumination.

apparatus should be thoroughly washed in warm water with soap and soda and then in large quantities of plain water.

✓ The hands should be

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always when performing post-mortem examinations on animals. Any wound received in the laboratory should at once be treated with tincture of iodine or some other disinfectant such as Dettol. When work is discontinued, the hands should be bathed in 2 per cent. lysol, 15 per cent. Dettol, or other disinfectant, and well washed with soap and water.

✓ The face, particularly the eyes, nostrils and mouth, should be carefully protected from all contact with contaminated material. Mouth suction should not be applied directly to pipettes for measuring fluids containing bacteria. If teats cannot be employed, suction should be applied through a rubber tube fixed to the end of the pipette. No food should be eaten in the laboratory. Gummed labels should never be moistened with the tongue.

No reasonable person attempts to prevent smoking in the laboratory, but it should not endanger the smoker. A pipe is safer than a cigarette. It should not be laid down so that the mouth piece is in contact with the bench. A cigarette should never be laid on the bench. If the mouth part projects over the edge, the bench will be burned; if the cigarette is reversed, the mouth may become infected.

Students should remember that a number of careful and experienced bacteriologists have died of infections contracted in their laboratories.

trons, thus serving the same purpose as glass lenses in relation to light rays. The image may be received on a photographic plate or on a fluorescent screen which renders it visible. Since electrons are easily deflected from their course by molecules of gas, the whole system must operate in a high vacuum. The electron microscope is so costly and complicated that it is unlikely to replace the normal microscope except where very high magnifications are required.

When using the ordinary microscope, certain rules must be observed. For examining stained bacteria in films or sections, the oil-immersion lens is generally employed. With this the condenser and plane mirror are used, and the diaphragm should be widely opened unless the light is excessively bright; where an artificial light is employed, intensity may be lessened by withdrawing the microscope to a greater distance. The mirror should be carefully adjusted and the condenser focused so as to secure uniform illumination of the field. For studying living bacteria, a dry lens (1/6-inch) is preferable. With this the best illumination is obtained when the concave mirror is used without a condenser and the diaphragm is nearly closed. If the light is too intense, the bacteria cannot be seen.

By racking the microscope tube down carelessly it is easy not only to ruin the preparation but also, a much more important thing, to dislocate or fracture the front lens of the objective. To avoid this catastrophe, the following rules must never be departed from in focusing the microscope. The coarse adjustment must be used to lower the objective to within its working distance from the object. This must be done by direct observation of the distance between the upper surface of the slide or cover glass and the objective, the observer's eye being level with the slide. This adjustment must never be made with the eye at the ocular. When the oil-immersion lens is used it must be lowered cautiously into the drop of oil, and then a little further, so that the drop is seen to be flattened out; it must never come in contact with the cover glass. The eye is now applied to the ocular and the mirror adjusted to give good illumination. The coarse adjustment is worked so as

For the study of certain bacteria, particularly the spirochetes, which are practically invisible in the living condition with ordinary illumination, a special form of condenser is employed. In this the rays of light are thrown so that none of them objects are visible seen in outline only. "ultra-

microscopic" particles appear as bright points of light. This type of illumination is similar to that which renders visible the moles which are seen when a bright beam of light enters a dark room. In using a dark ground condenser, it is essential to have a layer of oil between the condenser and the lower surface of the object glass and also to have a very bright illuminant, such as an electric arc or a Pointolite lamp. With most types of dark ground condenser, it is necessary to have a "funnel stop" which is dropped within the body of the objective, or else to use an objective with a built-in diaphragm, in order to cut off all direct rays of light.

The magnification with sharp definition attainable by the ordinary microscope is limited partly by the wave length of light and partly by the physical properties of glass used as a refracting medium in lenses. By employing mirrors in place of lenses, the difficulties arising from the use of these can be eliminated and the magnification attainable is then limited only by the wave length of light. There appear to be good prospects of the reflecting microscope, which is now in the experimental stage, becoming generally available in the near future. With this instrument one should be able to attain higher magnifications than are possible with the best ordinary microscope and without loss of definition.

When, instead of light, a beam of electrons is used, as in the electron microscope, the limitation imposed in optical microscopes by the wave length of light is eliminated and a magnification of $\times 100,000$ can be obtained.

In the electron microscope, the object is mounted on a thin collodion membrane, supported on a metal gauze screen, instead of on a glass slide, a beam of electrons takes the place of a beam of light and magnetic fields are used to refract the beam of elec-

beyond the cover slip, nor too small, for if it is bubbles will form. If the preparation has to be kept for some time it may be sealed, so preventing evaporation, by running around the edge of the

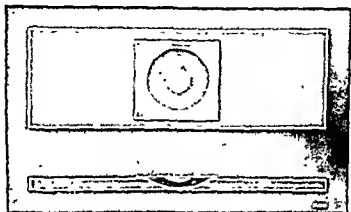


FIG 5—HANGING DROP PREPARATION USING HOLLOW GROUND SLIDE.

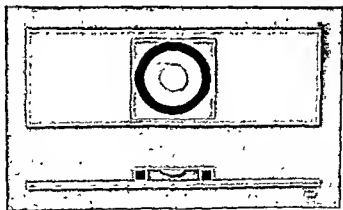


FIG 6—HANGING DROP PREPARATION USING ORDINARY SLIDE AND RING

cover slip a thin glass rod dipped in melted paraffin wax. At first it will be found difficult to see the bacteria on account of their transparency. Artificial light, no condenser, concave mirror and

to elevate the objective very slowly until the object is seen. If it is passed unobserved, it is necessary to repeat the operation from the beginning. The objective must never be racked down with the eye at the ocular as this may ruin the lens owing to its coming in contact with the slide. Once the object has been found, fine focussing is done with the fine adjustment, the mirror is arranged and the condenser focussed so as to get critical illumination. The fundamentals are: (1) Never rack down unless the objective is under direct observation; (2) always "find" the image of the object by racking upwards with the coarse adjustment, never downwards; (3) never use the fine adjustment to find the image, only to get the best possible focus.

In using the microscope, both eyes should be kept open, as this causes less strain than when one is closed. At first the two images will cause trouble, but after a time one becomes accustomed to ignoring what is seen with the eye not applied to the microscope, especially if the bench is dark in colour and not too brightly illuminated.

Bacteria may be observed microscopically either unstained in fluids or stained in films or sections. The method of examining living or dead bacteria, unstained, will first be described. For this purpose we may make a hanging-drop preparation, using either a hollow ground slide or an ordinary slide with a glass, vulcanite, rubber or metal ring about 2.0 cm. in diameter by 0.3 cm. high. In the latter case, the ring is held by a forceps and dipped in melted vaseline and is then dropped on the centre of a slide. The hollow ground slide is prepared by running melted vaseline in a ring round the hollow. A drop of the broth culture, or other fluid containing the bacteria, is placed in the centre of a cover slip and the prepared slide lowered over it until contact is made between the vaselined surface and the cover slip. The slide may now be inverted and the cover slip gently adjusted, so that the drop hangs suspended in a chamber sealed with the vaseline: this prevents the drop from drying. An alternative method is to place a drop of the fluid on a slide and to drop a cover slip on it. The drop should be neither too large, in which case some of it will flow out

is to rub the slides well with a soapy cloth and then to polish without washing off the soap. In the case of a fluid, whether culture medium, urine, cerebro-spinal fluid, or other body fluid, it is only necessary to place a drop of this in the centre of the slide and spread it slightly with the platinum loop. If the bacteria and cells in a fluid such as urine are few, they may be concentrated by centrifuging, films being made from the deposit. Films of pus may be spread with a platinum loop. A very thin, even film is, however, desirable, and better preparations may be secured by dipping a cotton wool swab in the pus, expressing the excess

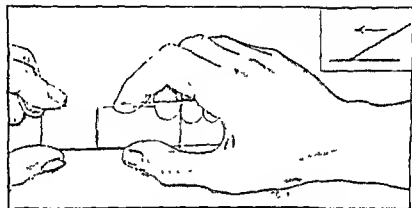


FIG 7 —METHOD OF SPREADING BLOOD FILM

against the wall of the tube and then "dabbing" the swab on the slide several times, each time on a fresh surface. If the pus is very thick, or contains small nodules of caseous material, the same procedure may be adopted as just described with caution. A fragment is placed tightly down the nodules, and are then drawn apart. Blood films are made by placing a small drop of blood on a slide towards one end. The edge of another slide is moved along the surface of the first until it comes in contact with the drop, when the blood spreads out between the two. The second slide is held at an angle of about

diaphragm almost closed will assist. It may help the beginner to focus the bacteria if a mark is made with a grease pencil at one side of the lower surface of the cover slip before the preparation is mounted. If this is brought sharply into focus and the slide then moved, so that the drop is below the objective, the bacteria themselves should at once be visible. It is necessary to determine whether these are motile or not. True motility must be distinguished both from Brownian movement, which is due to molecular bombardment, and is also seen in minute, lifeless particles, and from the flowing of the bacteria owing to currents set up by uneven heating. The best test is to observe carefully two bacteria lying apart. If these move is necessary to from unusual activity of non-motile organisms or from sluggishness of those normally motile. Excessive Brownian movement or sudden changes in surface tension, such as occur when bacteria from a and once a rich, able ses, shows of locomotion. The may be investigated by con- them in a suitable medium and at a suitable temperature.

As very little can be discovered concerning bacteria in unstained preparations, apart from the question of motility and of reproduction, the methods of staining bacteria are of great importance. For satisfactory preparations, clean slides, free from grease, are essential, as the least trace of any oily substance will prevent a drop of water spreading in a thin, even film. The slides may be treated with a solution of potassium bichromate in dilute sulphuric acid, followed by washing in water and drying, or may be heated strongly in the Bunsen flame. A simple alternative method

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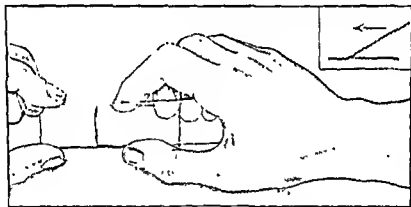


FIG 7—METHOD OF SPREADING BLOOD FILM

against the wall of the tube and then "dabbing" the swab on the slide several times, each time on a fresh surface. If the pus is very thick, or contains small nodules of caseous material, the same procedure may be adopted as is used with sputum. A fragment is placed on the slide and another slide applied to this; the two are tightly pressed together, rubbed over one another, so breaking down the nodules, and are then drawn apart. Blood films are made by placing a small drop of blood on a slide towards one end. The edge of another slide is moved along the surface of the first until it comes in contact with the drop, when the blood spreads out between the two. The second slide is held at an angle of about

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guished from molecular motion, which is due to particles, and is caused by uneven heating.

The best test is to observe carefully two bacteria lying close together. If these move in the same direction, they do not exhibit true motility. It is necessary to guard against wrong conclusions which may arise from unusual activity of non-motile organisms or from sluggishness of those normally motile. Excessive Brownian movement or sudden changes in surface tension, such as occur when bacteria from a solid medium are suspended in water, may simulate motility, and

and at a suitable temperature.

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alcohol and ether but, more commonly, the film is fixed by passing it three or four times slowly through the Bunsen flame, film side upwards. Overheating will be avoided if, during heating, the slide is held between the finger and thumb, and if, after heating, the temperature of the under surface of the slide is tested by holding it against the sensitive part of the back of the hand which lies between the base of the thumb and forefinger. When cool, and not before, it is ready for staining.

Since stains are very liable to soil the bench, staining should be done on a rack, over a sink. Such a rack consists of two parallel glass or metal rods arranged so as to be absolutely horizontal and about 2 inches apart. These may be mounted in plasticine so that they are easily adjusted and readily removed. On these the slides rest during staining, and if heat is required it may be conveniently applied with a small torch made by moistening a cotton swab on a wire with spirit. Soiling the fingers is avoided by using forceps for lifting the slide.

The stains most commonly used in bacteriology are solutions of basic aniline dyes in water. Many types of bacteria can be satisfactorily stained by flooding the fixed film with the stain which is allowed to act for a period ranging from a few seconds to several hours, depending on the intensity of action of the particular stain used and the type of bacteria. After staining, the slide is thoroughly washed with tap water and dried by blotting. For this purpose fluffless blotting paper or filter paper should be used. A bed of several sheets of the paper is made on a smooth table, the slide is laid on this, film side upwards, and is covered with a pad of several sheets of the absorbent paper which is pressed into contact with the film with the edge of the palm. The paper must not be rubbed on the slide as this would remove the film. The same sheet of paper should not be used for drying another preparation since minute portions of film can thus be transferred from one slide to another. The film is then dried by waving the slide over the flame of a Bunsen. It must be completely dry before immersion oil is applied to it.

In the case of some bacteria which stain with difficulty, the

30° with the first and pushed slowly over its surface. The blood is drawn along with the upper slide and produces a thin film on the surface of the lower.

To make a film from a culture on solid medium, a drop of water is placed on a slide with the platinum wire. The wire, after sterilisation and cooling, is allowed to touch the culture, a small portion of which adheres to it. The wire is then rubbed in the drop which is spread out to form a film. Great care must be taken to avoid adding too much culture to the water as the chief error likely to occur is to make the suspension far too thick: it should be but faintly opalescent. Beginners sometimes find it hard to understand why tap water, containing bacteria, may be used in preparing films, but must not be added to cultures. Let us suppose that the water contains 100 bacteria per ml. A platinum loopful added to a tube of broth would probably contain two bacteria which might grow in the broth when it is incubated and so contaminate the culture. But if a loopful were used for the preparation of a film, the latter would probably measure about 4 sq. cms. The field seen with an average oil immersion lens measures about 0.0002 sq. cm., that is $1/20,000$ of the area of the film. From this it follows that one of the two water bacteria introduced would be seen in the examination of something like 10,000 fields. The chances against any bacteria present in the water being seen in the microscopic examination of a film are, then, very remote.

Films, however prepared, must be dried and then fixed. Drying is best carried out at air or body temperature (in an incubator), protected from dust. If the amount of fluid taken is large the film will take a long time to dry, and there is a great temptation to hasten drying by heating, which is one of the commonest causes of the unsatisfactory films frequently made by students. The most that can be allowed is to wave the film about, high above the Bunsen, where the fingers are not uncomfortably heated.

When the film is dry it is fixed, so that the bacteria and cells are made to adhere firmly to the slide. This may be accomplished by the use of absolute alcohol or a mixture of equal parts of absolute

alcohol is very prone to remove some of the stain, excess of water may be removed by careful blotting and dehydration of sections may be accomplished with aniline oil, aniline-xylol, and then xylol. A less perfect, but sufficiently good method for most purposes, is to blot the section, allow to dry in air and clear in xylol. It is then mounted with a cover slip (No. 1) in Canada balsam.

In the directions for carrying out various staining methods given below it may be assumed, unless the contrary is stated, that films are dried and fixed by heat previous to staining and that, at the end of the operations described, the film is dried by blotting followed by moderate heat

Indian Ink and Nigrosin

One of the quickest and simplest methods of determining the morphology of bacteria is not, strictly speaking, a staining method. In it bacteria are rendered visible unstained against a dark background. This background may be composed of Indian ink (Higgin's waterproof ink is satisfactory) or of nigrosin.

To a drop of a 1 : 2 dilution of the ink or of a solution of nigrosin on a slide is added a loopful of the material containing the bacteria. An even suspension of these is made in the fluid which is spread out with a platinum wire. The film so formed is allowed to dry and then the bacteria are fixed by passing the slide through a flame. The preparation is examined with an oil-immersion lens. It is an advantage rather than a disadvantage to have a somewhat uneven film as a portion may be selected for examination presenting the best contrast between the dark background and the unstained bacteria

An alternative method is to prepare a film of bacteria in the usual way, fix and, if desired, stain and then to spread over the dry film a drop of Indian ink or nigrosin solution, spreading being done with another slide in the same way as a film of blood is spread

Both Indian ink and nigrosin have disadvantages. The former

often applied successively to the same preparation. In some cases, certain portions of a bacterium have a special affinity for one stain, others for another, so that, by staining first with one and then with the other, a contrast in colour between different portions of the organism is obtained. The special staining methods, such as Neisser's, used to demonstrate the granules of *C. diphtheriae*, are examples of this type of double staining. With another type, the basis is not the ease with which a portion of a bacterium can be stained but the fastness with which certain organisms, once stained, retain the stain. After treatment with a stain of one colour the slide is treated with alcohol, acid or other decolorising agent which removes the stain from some species of bacteria but not from others. Then the film is counter-stained, that is stained with a stain the colour of which contrasts with that first used. The organisms decolorised by alcohol or acid are stained by the second stain. Examples of this form of double staining are Gram's and the Ziehl-Neelsen stains.

The film, after staining, washing and drying, may be mounted in Canada balsam with a cover-glass (No. 1) but, unless permanent preparations are required, it is more usually examined unmounted, a drop of oil being applied to it and the oil-immersion lens used. If it is desired to keep such a film, the oil may be removed with xylol and the film stored dry, protected from dust.

such sections are described in textbooks of histology and pathology. To stain a paraffin section, it is necessary to remove the paraffin completely with xylol and this in turn with alcohol. After washing with water, the section may be stained as described for films, but it is advisable to increase the time given to each operation, especially decolorisation where that is necessary. As the usual histological process of dehydration with absolute

Neutral Red

Stain films for 2 to 5 minutes and wash with water. Used chiefly as a counter-stain in Gram's method.

Neutral Red and Carbol Fuchsin

Stain films $\frac{1}{2}$ to 1 minute and wash with water. One of the best counter-stains in Gram's method particularly for the identification of gonococci in pus.

Carbol Thionin Blue

Used chiefly for sections. Stain for 10 to 15 minutes. After washing in water, treat for a few seconds with 1 : 1000 acetic acid and again wash in water. Bacteria, which appear purple, contrast well with the blue tissue cells.

Bismarck Brown

Not used for films except in Neisser's method, and as a counter-stain in Gram's and the Ziehl-Neelsen methods. Stain films or sections 5 minutes and wash in water. Does not overstain cells.

Bacteria Grams Pos. & Neg.
Double Staining

Gram's Method

first steps in its identification. In Gram's method the organisms are first
2) are the
3) alcohol
are Gr

lose the violet colour. A counterstain is used in order to stain Gram negative organisms and any cells present. A complex has been isolated from Gram positive bacteria which has not been found in Gram negative bacteria. This complex, which consists of ribonucleic acid, protein and magnesium, may be the cause of

gives a granular background and is liable to contain bacteria: the latter is a powerful decoloriser, removing stain from bacteria unless the period of contact before drying is very short.

Simple Stains

Below are notes on the use of the more common simple stains, the formulæ of which are given in Chapter XLVIII. Since many of these stains are liable to precipitate when kept, it is good practice always to filter a stain on to a slide. Small funnels fitted with filter papers may be used for this purpose.

Löffler's Alkaline Methylene Blue

✓ Stain films for 5 minutes, sections for a rather longer time, and wash with water. This stain is particularly useful for staining films for the detection of *C. diphtheria*. An old stain which has developed polychromatic properties is very good for this purpose. After washing with water, counterstain with 1% fast green for 1 minute, wash with water, and then use it should be followed, after washing, with 1:1000 acetic acid solution which removes more colour from the tissue cells than from the bacteria. Wash well with water after the acid treatment.

Crystal (or Methyl) Violet

A very intense stain. Stain films for 30 seconds or less and wash with water. Used mainly in Gram's method.

Carbol Fuchsin

Too intense to use as a simple stain as even 2 or 3 seconds treatment overstains. Used mainly in the Ziehl-Neelsen method.

Dilute Carbol Fuchsin

✓ Stain films for $\frac{1}{2}$ to 1 minute and wash with water. Used chiefly as a counter-stain for films in Gram's method.

until fresh alcohol removes little or no violet from the film. Only practical experience will teach how much washing with alcohol is correct. Films require

(7) The film is then counter-stained. The object of this is to render visible Gram negative bacteria and cells from which the alcohol has removed the violet colour. The counter stain must contrast in colour with the violet of Gram positive bacteria and should not be too intense. Dilute carbol fuchsin, neutral red, neutral red with carbol fuchsin or Bismarck brown may be used, the times of staining being the same as for simple staining given above. Of these the one most commonly used, dilute carbol fuchsin, is probably the worst, as the contrast between it and the violet is not always very marked. The combination of neutral red and carbol fuchsin is a good counter-stain. When attention is directed mainly to Gram positive bacteria, and the Gram negative bacteria and cells are of minor importance, Bismarck brown is very useful. For the detection of gonococci in films of pus, Pappenheim's stain is recommended as the counter-stain

8. Wash with water.

For sections, the method as far as step 6 is only slightly modified. In steps 1 and 3 double the times given should be allowed. Decolorisation (5) requires a very much longer time than for films. It is advisable, after decolorisation, to treat the section with acetone for a few seconds as this removes crystals which occasionally prove troublesome. Counter staining may be performed with 1 per cent. alcoholic eosin for 2 or 3 seconds or with Bismarck brown for 5 minutes. Dilute carbol fuchsin should not be used.

Excellent practice is afforded in the technique of Gram's method by making films of a mixture of a staphylococcus and Bact. coli. After some time it will be found possible to produce a film in which all the cocci are violet and all the bacilli red or brown, depending on the counter-stain used. In examining preparations stained with Gram's method it is of importance that the dia-

the characteristic reaction to the stain of Gram positive organisms. It can be removed from the bacteria by treating them with bile and may be restored to the organisms, again rendering them Gram positive. It cannot be so applied to Gram negative bacteria.

Gram's method is one of the most difficult of those commonly employed, but the technique must be thoroughly mastered before any advances can be made in bacteriology. The difficulty most usually encountered is in the decolorisation. Most bacteria are definitely positive or negative but, with a few, the result may be doubtful since they appear positive if decolorisation is curtailed and negative if it is prolonged. The real difficulty here is not in the staining, but in the preparation of the film. If this is uneven, thick in one part and thin in another, a good result is practically impossible since, if the thin parts are correctly treated with alcohol, the thick portions are not sufficiently decolorised, while, if attention is paid to these, the thin parts will be over-decolorised. Most

unstable stain which only keeps for a few days, and a weaker iodine solution were employed, but the one here described, that of Jensen, is probably the simplest and most reliable.

- (1) Stain with crystal violet 1 minute. *Crystal violet*
- (2) Rinse rapidly with water.
- (3) Flood with Lugol's iodine and leave the solution on the slide for 1 minute.
- (4) Pour the iodine solution off the slide and shake it so as to free it as completely as possible of the solution.
- (5) The film is then rapidly flooded with alcohol, the flooding being twice repeated to remove all traces of iodine without delay. It is then covered with alcohol and rocked, as is a photographic plate during development. The alcohol takes up some of the violet colour and, when this occurs, it is poured off and replaced with fresh. The whole washing process is repeated several times

if the pink colour returns, the acid alcohol is replaced. It may be necessary to alternate acid alcohol and water several times before the film has been sufficiently decolorised.

Tubercle bacilli appear bright red and other bacteria and cells the colour of the counter-stain used.

Sections are stained in the same way as films

The method may be used for other acid-fast bacteria but, since many of these are acid- but not alcohol-fast, neither alcohol (step 5) nor the alternative, acid alcohol, should be used. The use of alcohol is important when tubercle bacilli are being looked for as it decolorises many of the bacilli likely to cause confusion. A good rule to remember when using the Ziehl-Neelsen method to demonstrate tubercle bacilli is that it is practically impossible either to over-stain with fuchsin or to over-decolorise with acid or alcohol.

Stains for *C. diphtheria*

In the identification of *C. diphtheria*, it is always advisable to use Gram's stain since the organism is Gram positive, a point of considerable value in identification, but this stain obscures the

as it
the

granules.

In addition a number of other stains are commonly used. All of these demonstrate the granules.

Neisser's Stain

1. Neisser's methylene blue—3 minutes.
2. Wash rapidly with water.
3. Counter stain. A great variety of counter stains are used:
 - (a) Bismarck brown—3 minutes
Since this stain is not intense, it does not tend to obscure the granules when these are poorly developed.
 - (b) Chrysoidin—1 minute.

phragm of the microscope should be widely open as the distinction between Gram positive and negative bacteria is more apparent vary in the degree to decolorisation. This bacteria but also on the age of the culture. Young cultures, especially during the stage of active multiplication, tend to be strongly Gram positive, while cultures 48 hours or more old tend to become increasingly readily decolorised.

Ziehl-Neelsen-Method-for-Tubercle Bacilli

1. The fixed film is covered with the stain (carbol fuchsin) and heat applied below the slide either with a small flame of the Bunsen, a spirit lamp, or spirit torch. The heating should be sufficient to cause steam to rise, but boiling the stain must be avoided. Staining should last for at least 10 minutes, heat being applied occasionally. Care must be taken to prevent the stain drying on the slide, fresh stain being added if necessary.

2. Wash well in water.

3. Flood the slide with 20 per cent. sulphuric acid in water and leave on for 1 or 2 minutes. In the acid the pink colour changes to yellow or brown.

4. Wash in water. The pink colour may return. If so steps (3) and (4) are repeated as many times as necessary, until the film has not more than a faint pink tinge.

5. Wash in alcohol 1 minute.

6. Wash in water.

7. Counter stain. Löffler's methylene blue applied for $\frac{1}{4}$ minute is commonly used but this is not a good counter stain as, with it, cells are so intensely stained as to render difficult the detection of small numbers of tubercle bacilli. Recommended alternatives are:

Malachite green	.	.	.	1 minute
Bismarck brown	.	.	.	5 minutes

8. Wash with water.

Steps 3 and 5 may be replaced by treatment with acid alcohol. After treatment for 5 minutes, the film is rinsed with water and,

if the pink colour returns, the acid alcohol is replaced. It may be necessary to alternate acid alcohol and water several times before the film has been sufficiently decolorised.

Tubercle bacilli appear bright red and other bacteria and cells the colour of the counter-stain used.

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Since this stain is not intense, it does not tend to obscure the granules when these are poorly developed.

(b) Chrysoidin—1 minute.

(1) Safranin—2 minutes.

(2) Löffler's methylene blue—3 minutes.

The combination of the two methylene blues is used by some bacteriologists as the routine stain for the detection of *C. diphtheriae* in cultures. It reveals both the morphology of the organism and the granules. The results are improved by treating the film with Lugol's iodine for 1 minute and washing with water between steps 2 and 3.

Albert's Method (modified)

1. Toluidin blue-malachite green—5 minutes.

2. Do not wash off. Blot, dry.

3. Lugol's iodine—1 minute.

4. Do not wash off. Blot, dry.

This is a good stain for granules which are stained blue-black, the bodies of the bacilli being blue.

Pugh's Stain

This stain is used in a peculiar way.

1. Place a drop of the stain on the fixed film.

2. Cover with No. 1 cover glass.

3. Examine with oil-immersion lens.

Granules are blue; the bodies of the bacilli are almost unstained.

Methods of Demonstrating Spirochetes

Some spirochetes such as *C. vincentii* can be stained with simple stains; rendered visible

✓ *Tr. pallidum*, require special methods.

Indian Ink or Nigrosin

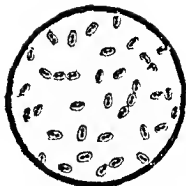
By the use of one of these, spirochetes may be rendered visible, standing out unstained against a dark background.

✓ Fontana's Method

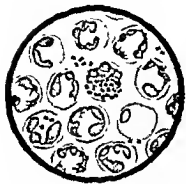
This is probably the most satisfactory method of demonstrating spirochetes in films. It is a silver impregnation method, the



1



4



2



5



3



6

BACTERIA AS SEEN WITH THE OIL-IMMERSION LENS.

1 Staphylococci in pus

2. Gonococci in pus.

3. Tubercle bacilli in sputum

4 Pneumococci stained to show capsules

5. *CL tetani* stained to show spores

6. *B anthracis* stained to show spores.

(c) Safranin—2 minutes.

(d) Löffler's methylene blue—3 minutes.

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1. Place a drop of the stain on the fixed film.

2. Cover with No. 1 cover glass.

3. Examine with oil-immersion lens.

Granules are blue; the bodies of the bacilli are almost unstained.

Methods of Demonstrating Spirochætes

Some spirochætes, such as *Sp. vincentii*, can be stained with simple stains; others, such as those of the relapsing fevers, can be rendered visible by Leishman's stain; but a number, including *Tr. pallidum*, require special methods.

Indian Ink or Nigrosin

By the use of one of these, spirochætes may be rendered visible, standing out unstained against a dark background.

Fontana's Method

This is probably the most satisfactory method of demonstrating spirochætes in films. It is a silver impregnation method, the

spirochaetes being rendered visible by the deposition of metallic silver on their surfaces, which makes them appear much coarser than they really are.

1. Dry film without heat.
2. Fix in acid formalin solution for about 1 minute, the reagent being renewed three times during this period.
3. Wash well with water.
4. Treat with mordant for $\frac{1}{2}$ minute, gentle heat being applied.
5. Wash well with water.
6. Treat with silver solution for $\frac{1}{2}$ minute, gentle heat being applied.
7. Wash well with water.

The spirochaetes are stained dark brown or black.

The silver solution should not be kept for more than a few hours as, if kept for a longer time, a brown precipitate may form and this is a most violent explosive.

Levaditi's Method

This is also a silver impregnation method but, since it is applied to blocks of tissue and not to individual sections, it is not here described. The treated tissue is embedded in paraffin and sections are cut. These, when fixed to slides, merely require to be freed from paraffin wax with xylol and mounted in Canada balsam or examined in oil without a cover glass. Spirochaetes appear black and tissue cells brown. It is almost the only satisfactory method for the demonstration of spirochaetes in organs removed at post-mortem examination.

Capsule Stains

Hiss's Method

This method is most successful when applied to films freshly made from infected body fluids. It demonstrates the capsules of pneumococci very well in the blood or peritoneal fluid of an

(6a) Spread a thin film of nigrosin over the film with another slide.

(7a) Dry rapidly.

In this modification, the red spores contrast with the unstained bodies of the bacilli.

Other Stains

Leishman's Stain

This stain is particularly useful for the demonstration of organisms such as spirochaetes, malaria parasites and trypanosomes in blood, and of Past. pestis in blood and bubo fluid. Thin, even films are prepared and allowed to dry without heat.

1. Cover with stain. Leave 1 minute. This fixes the film.

2. Add four times the volume of distilled water, mix, allow to act for 10 minutes.

3. Wash thoroughly in water.

Pappenheim's Stain

Stain films for 1 minute and wash off with water. This stain is

p
b

nuclei of cells are a blue-green colour. This stain may be used as a counter stain in Gram's method. The red gonococci contrast with the purple staphylococci and streptococci and the blue-green cells.

Fluorescence Microscopy

If bacteria are stained with fluorescent dye and examined microscopically, ultra-violet rays being used instead of ordinary light, they fluoresce and so become visible as bright objects in a dark field.

The method has been used chiefly for the detection of tubercle bacilli in films. Films, which must be thin, are best made on slides of special glass permeable to ultra-violet rays. They are fixed as usual, stained in auramine for 15 minutes at air temperature,

1. Crystal violet with heat— $\frac{1}{2}$ minute.
2. Wash off with a 20 per cent. solution of copper sulphate: do not use water.
3. Blot and dry.

tained, it is important that only the minimum amount of heating be employed for fixation and during staining and that the shortest possible time, preferably not more than 5 seconds, should elapse between the addition of the copper sulphate solution and the complete drying of the film.

Nigrosin Method

1. Crystal violet or carbol fuchsin with heat— $\frac{1}{2}$ minute.
2. Blot and dry.
3. Spread over this film a thin film of nigrosin, using another slide as in preparing a blood film.
4. Dry.

Nigrosin decolorises the capsules so that the bodies of the bacteria are seen coloured with the stain used and surrounded by unstained capsules.

Spore Stains

Möller's Method

1. Carbol fuchsin with heat—10 minutes.
2. Wash with water.
3. Decolorise with 5 per cent. solution of sodium sulphite— $\frac{1}{2}$ minute.
4. Wash with water.
5. Counter stain Löffler's methylene blue—1 minute.
6. Wash with water.

Spores are stained bright red, protoplasm blue. The optimum time for step 3 must be determined by trial as spores of some bacteria are more easily decolorised than those of others. An alternative method is to substitute for steps 5 and 6

(5a) Blot and dry.

CHAPTER III

STERILISATION

In the cultivation of bacteria we have two cardinal points requiring attention—the preparation of food material and the exclusion of extraneous organisms. Of these the latter is of such fundamental importance that it will be dealt with first. Sterilisation is the name given to the process by which bacteria are destroyed. It may be accomplished in a variety of ways, each of which has its uses in the laboratory.

1. Sterilisation by Heat

This is a simple and satisfactory method which is capable of being employed in many ways. Contaminated paper or dressings, bodies of infected animals and the like, are rendered harmless by burning. Small pieces of apparatus, such as platinum wires and forceps, the flame capillary


flame. When the flame begins to become yellow, which indicates that fusion is about to commence, the glass is certainly sterile.

Larger articles of glass—test tubes, flasks, Petri dishes—as well as cotton wool and paper, cannot be sterilised in this way. For

exposure at 140 to 150 °C for 3 hours is preferable, as this causes less destruction of organic material such as paper or cotton wool. Glass vessels must be put into the chamber before it is heated,

washed with water and decolorised with a special acid alcohol applied twice for $1\frac{1}{2}$ minutes on each occasion. Finally they are well washed and dried. No counter stain is used. ✓ Since glass filters off much of the ultra-violet radiation, the condenser should preferably be made of quartz, but quite good results may be obtained with an ordinary glass condenser. The usual high power, dry objective ($1/6$ inch) and eye-piece are used.

The rays from a mercury vapour lamp, filtered free of visible light by passing through Wood's glass and from red and infra-red rays by passing through a 4 per cent. solution of copper sulphate, are directed by the mirror and condenser through the stained film. Any tubercle bacilli present fluoresce and so become visible when magnified by the normal optical system. They show up as luminous rods in a dark field. Since the $1/6$ inch objective is used in this method, a much larger area of film and, therefore, a much larger amount of sputum can be examined in a given time than by the usual method. ✓ Hence it is less likely that small numbers of tubercle bacilli in a specimen of sputum will be overlooked by the fluorescent method than by the ordinary staining methods. On the other hand, since some objects other than tubercle bacilli may fluoresce when stained in this way, a positive result should be confirmed in a preparation stained by the Ziehl-Neelsen method.



of the water. The steamer is used for sterilisation of media or other material. An exposure to a temperature of 100° for a few minutes is sufficient to kill all non-sporing bacteria, but one must remember that some time is needed for steam to heat the fluid contained in a large flask to its own temperature. For this reason vessels of media are generally left in the steamer for 20 to 30 minutes after the water has commenced to boil. Spores are not all destroyed by steaming but, by the method



FIG 9—STEAM STERILISER
(ARNOLD PATTERN)
(Messrs Gallenkamp)

the vessel being kept at air temperature between each exposure. The principle is that the first steaming kills all vegetative forms. In the interval between this and the next exposure any spores present assume the vegetative form, and these are killed at the second steaming. The third heating is an additional precaution.

The same intermittent method may be applied at lower temperatures than that of steam, e.g. 60° . In this case the number of exposures is usually increased to five or six. The method

must be made of pasteurisation. This process, which is chiefly used for the treatment of milk, consists in keeping the fluid at a temperature of 63° to 65° for half an hour. While not sufficient

and left to cool in it, as any rapid change in temperature is very liable to crack glass.

Moist heat is a much more efficient agent in killing bacteria than is dry heat. It can be applied by boiling, as is done for syringes, surgical instruments and other small pieces of apparatus. Five minutes' boiling will kill all non-sporing bacteria and the majority of spores, but for complete destruction of all bacteria 1 hour or more is required.

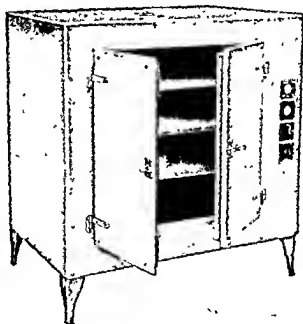


FIG 8—HOT AIR STERILISER
(Messrs. Baird and Tatlock (London), Ltd)

For larger apparatus a "steamer" is used. This, in its simplest form, is merely a large metal vessel provided with a lid and containing some water in the bottom. This water is boiled by means of electricity or gas, and from it steam rises and fills the vessel, escaping through a small opening near the top. The newer steam sterilisers, such as the Arnold, are an improvement on the old since steam can be raised in them in a few minutes. Flasks or other vessels rest on a perforated shelf situated above the level

of the water. The steamer is used for sterilisation of media or other material. An exposure to a temperature of 100° for a few minutes is sufficient to kill all non-sporing bacteria, but one must remember that some time is needed for steam to heat the fluid contained in a large flask to its own temperature. For this reason vessels of media are generally left in the steamer for 20 to 30 minutes after the water has commenced to boil. Spores are not all destroyed by steaming but, by the method



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must be made of pasteurisation. This process, which is chiefly used for the treatment of milk, consists in keeping the fluid at a temperature of 63° to 65° for half an hour. While not sufficient

to sterilise, this is sufficient to kill most of the pathogenic bacteria likely to occur in milk.

The next method is by the use of the autoclave, which is a very strong boiler with a lid which can be hermetically sealed. The autoclave, which must be provided with a pressure-gauge, a valve for releasing pressure and a safety valve, contains water in the bottom. The vessels are placed on a perforated shelf and the lid

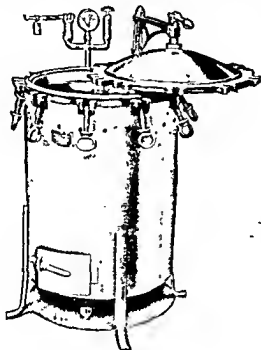


FIG 10.—AUTOCLAVE.
(Messrs. Baird and Tatlock (London), Ltd)

screwed down. Heat, provided by electricity or gas, is applied and when the water boils steam emerges from the valve. It must be allowed to flow for a considerable time in order to expel all air, for the pressure-gauge acts also as a thermometer, but its readings are unreliable unless all air has been expelled. This can be judged by leading the escaping steam by a rubber tube to a bucket of water. If there is no bubbling, all air has been expelled. The valve is closed and the pressure in the interior, as indicated

by the gauge, rises. The greater the pressure the higher is the temperature: 10 lbs. pressure per square inch is equivalent to 115°. An exposure to this temperature for 15 minutes will kill all known forms of life. This temperature should not be exceeded owing to the harmful effect of high temperature on many of the medium. After the requisite time has elapsed, heating is stopped, and the apparatus allowed to cool. The valve must not be opened until the internal pressure is the same as, or below, that of the outside air, as otherwise the fluids will boil violently and expel the plugs from the tubes or flasks. The valve should always be opened gradually before the lid is unscrewed in order to admit air to the vacuum in the interior. Before use it is essential to see that the apparatus contains sufficient water, both to avoid accident and also to prevent the steam from being superheated, since saturated steam is much superior in disinfecting power to superheated steam. Autoclaving is the quickest and most certain method of sterilisation.

never does good, but is frequently harmful.

12. Sterilisation by Chemical Means

Another method of sterilisation is by the use of chemical disinfectants. This has a small role in the preparation of media, but is extensively used in the laboratory for the disinfection of contaminated material, small pieces of apparatus and the hands. Of the commonly used disinfectants, lysol (2 per cent.) is probably the most useful and reliable.

13. Other Methods of Sterilisation

The passage of an electric current has been used as a method of sterilisation in some cases, but it is probable that the effect is due either to heat or to the liberation of chemical substances, such as chlorine, and not to electricity *per se*.

The foregoing methods all have for their purpose the killing of bacteria. The remaining method of sterilisation is one by which bacteria, whether dead or alive, are removed from fluids



FIG. 11.—STERILE
GRADUATED
PIPETTE.

free of bacteria. This process of sterilisation is considered in greater detail in Chapter VI.

Maintenance of Sterility

Having dealt with the methods of sterilisation, the next question is how to prevent bacteria from the air entering apparatus or fluids which are sterile. By far the most useful method is by plugging the mouths of tubes and flasks with non-absorbent cotton wool. Provided the cotton is dry, no bacteria can penetrate through its interstices. If it is damp, however, certain micro-organisms, particularly moulds, can grow through it and contaminate the material within. Test tubes and flasks, prepared to contain media, should be plugged fairly tightly with cotton wool and heated for 3 to 4 hours at 140° in the hot air steriliser. Petri dishes should be wrapped in paper or enclosed in metal boxes and sterilised in the same way.

The mouthpieces of graduated pipettes are plugged with cotton wool. The pipettes are placed in long thick-walled glass tubes, plugged with cotton wool, and sterilised in a hot air oven. The contained pipettes remain sterile until required.

CHAPTER IV

THE PREPARATION AND USE OF CULTURE MEDIA

(Refer to Chapter XLVIII for the formulae of the culture media mentioned.) 7.517 526

Artificial media for the cultivation of bacteria must supply carbon, nitrogen, hydrogen and oxygen, together with smaller amounts of many other elements, all in assimilable form.

A commonly used culture medium and one which forms the basis of many others is a clear, straw coloured fluid called, rather inappropriately, broth. Broth is made by dissolving in water, peptone (a mixture of digestion products of protein), which supplies the assimilable nitrogen as well as some of the minor constituents, and an extract of meat, which supplies both carbohydrates, used as fuel, and the rest of the minor organic and inorganic constituents required.

While the majority of pathogenic bacteria grow freely in broth, some of the more fastidious will not do so, either because the medium is lacking in some material essential for their metabolism

ble blood or of

which may be rectified by the addition of 0.1 per cent. of glucose.

One of the commonest and most undesirable of the prejudicial substances likely to be found in broth is copper which was formerly almost always present in commercial peptones and which, even now, is sometimes found in this material. In the preparation of broth by Wright's method, the peptone is added to the meat prior to filtration and any copper present in it com-

hines with the meat and is removed when the broth is filtered. In preparing media it is advisable, as far as possible, to avoid the use of metal vessels.

Additions may be made to broth which will render it either differential or selective. A differential medium is one which con-
tain an organism or group of
organisms which causes an alteration in the
medium so as to recognise the
presence, in the material cultured, of this organism or group of
organisms. By the addition of lactose and an indicator, broth is rendered a differential medium for lactose fermenting bacteria. If these are present, they ferment the lactose producing acid which changes the colour of the indicator, if absent, the indicator does not change.

A selective medium is one which inhibits the growth of all
bacteria except those of a particular type or group. A medium is

in it, the majority of other bacteria do not.

By suitable additions, a medium may be rendered both selective and differential. Lactose bile salt broth which contains an indicator is such a medium. Few bacteria other than intestinal types grow in it and, if lactose fermenting intestinal bacteria are inoculated into it, they grow freely and indicate their presence by a change in the colour of the medium. There are now almost a bewildering variety of selective media among which are those containing tellurite (permitting the growth of *C. diphtheriae* and suppressing that of most other organisms), gentian-violet (permitting the growth of streptococci and suppressing that of many other organisms) and the large number designed to permit the growth of intestinal pathogens (such as *Salm. typhi*) but not the much commoner *Bact. coli*.

The chief disadvantage of broth as a culture medium is that the majority of bacteria inoculated into it grow freely and that it is,

therefore, almost impossible to obtain a pure culture (a culture of a single type of bacterium) when it is inoculated with a mixture of organisms such as is commonly found in infected material. A further disadvantage is that, although some bacteria produce a characteristic type of growth in the medium, this is much less useful as an identifying feature than is the type of colony produced by the growth of bacteria on the surface of a solid medium.

Broth may be converted into a solid medium by making it into a jelly by the addition of gelatin or agar. Of these agar is far more commonly used. Bacteria grow in the depth of or on the surface of nutrient agar in virtue of the nutrients present in the broth from which it is made. Agar is sufficiently solid to prevent the movement of motile bacteria except on the surface when this is moist. The result is that, when a bacterium is deposited in or on agar and the latter is incubated, the bacterium multiplies producing a mass visible to the naked eye, known as a colony. If several types of bacteria are so deposited, each produces a separate colony from which pure cultures of each may be obtained.

The size and form of the colonies produced by different species of bacteria differ so widely as to be valuable characteristics by which they may be identified.

Agar may be enriched or converted into a differential or a selective medium in the same way as broth.

Reaction of Media ✓

As most bacteria live in the body fluids, in order, therefore, to get an essential that the initial reaction of the medium should approximate to that of blood.

The reaction of the medium should be ascertained with sufficient accuracy to permit of the use of different colours at different pHs. The most common indicator is phenolphthalein, the reaction of which is from acidity through neutrality to slight alkalinity. The reaction of a

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clarification (K) discs and negative pressure, is both quicker and more satisfactory.

Filtration of solid media in the liquefied state is usually carried out through filter paper but, if slight turbidity is not objectionable, filtration may be much more quickly accomplished through a bed of cotton wool in a funnel.

To obtain the clearest media, white of egg, beaten up with a little water, is added to the medium at a temperature of about 50° , two eggs being allowed for each litre of agar or gelatin. The medium is then steamed for 1 hour and filtered through paper. The coagulated albumin entangles the fine particles in suspension and, with them, is removed by the filter.

steam jacket or inside a steam steriliser.

Tubing of Media

The method of tubing media, whether broth, gelatin or agar in the liquid state or other fluid must be described, since it is of importance to be able to add to each tube the correct amount without the introduction of bacteria and without soiling the upper part of the tube into which the plug fits. To the end of a large funnel which is covered with a petri dish to reduce contamination from the air is attached, by a short length of rubber tubing, a glass tube drawn out to a moderately fine point. The rubber tubing is fitted with a pinch cock and the funnel is firmly held in a clamp. When it is filled with the medium, the cotton plug is removed from a sterile tube by holding it between the third and fourth fingers of the right hand and pulling the test tube away from it with a slightly screwing motion of the left hand. The drawn-out glass tube is inserted about half-way down the test tube and the pinch cock released with the right hand, until the test tube has received sufficient of the fluid, when the flow is stopped. The test

batch of broth is adjusted as follows. To 10 ml. of broth we add 0.5 ml. of a 0.02 per cent. solution of phenol red and titrate with N/10 NaOH until the colour matches that of one of the standard tubes, usually pH 7.4. To allow for the colour of the medium, a comparator box and blank tubes with broth should be used. By a simple calculation the amount of N or 10 N NaOH required to bring the whole bulk of broth to the selected pH is easily determined.

of two kinds: those which, when acted on by acids, form weak acid salts and those which, when acted on by alkalis, form weak alkaline salts. The reaction of a buffered solution responds only slowly, therefore, to the addition of acid or alkali. The buffers most commonly used in media are Na_2HPO_4 , NaH_2PO_4 and NaHCO_3 . Amino acids and other digestion products of protein also act as buffers.

Filtration

Clarity of a culture medium, while not essential, is for many purposes desirable, particularly in the case of fluid media. It is somewhat depressing to find a batch of broth which appeared crystal clear before sterilisation, cloudy after heating. The precipitate which forms consists mainly of phosphates and it is always found that, if a batch of broth is made more alkaline and subsequently heated, a precipitate forms. The best way of avoiding this is to adjust the reaction to the alkaline side of that required, steam and filter. If the final adjustment can then be effected by the addition of acid, little or no further precipitate will form on sterilisation.

Such a medium as broth can be cleared by filtering through folded filter paper in a funnel but, as the precipitate is very finely divided, the pores of the filter paper quickly become blocked and filtration is very tedious. Filtration through a Seitz filter, using

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Organisms, by their metabolism, may produce acids or alkalis in culture which tend, if permitted to accumulate, to alter the pH of the medium and so inhibit further growth. This is countered to some extent in most media by buffers. These are chemical salts of two kinds: those which, when acted on by acids, form weak acid salts and those which, when acted on by alkalis, form weak alkaline salts. The reaction of a buffered solution responds only slowly, therefore, to the addition of acid or alkali. The buffers most commonly used in media are Na_2HPO_4 , NaH_2PO_4 and NaHCO_3 . Amino acids and other digestion products of protein also act as buffers.

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The Use of Screw Cap Bottles

Screw cap bottles, introduced by McCartney, have much to commend them especially in small laboratories where, without them, much media in cotton plugged tubes may become unusable through evaporation. With these bottles, media only occasionally required may be stored for long periods without deterioration.

Round bottles of 1 oz. capacity may be used for broth, agar slopes (the medium being allowed to set with the bottles in a horizontal position) or other media and $\frac{1}{2}$ or even $\frac{1}{4}$ oz. (bijou) bottles for sugar media. A round bottle (1 oz.) is useful for storing sufficient solid medium for one plate, as well as saline, citrate and other solutions. Larger bottles (4, 6, 10 and 20 oz.) are used for the storage of broth, agar and other media.

The metal caps of the bottles are fitted with rubber washers and the round bottles are so strong that media may be autoclaved or steamed in them with the caps screwed tight.

Broth

There are many methods of preparing broth. The one described, that of Wright, has been found to be both simple and satisfactory.

Broth may be enriched and so made more suitable for the

Peptone Water

Peptone water is a solution of peptone and sodium chloride in water, sterilised by autoclaving. Its main use is to determine whether an organism produces indole but, for this purpose, it has no advantage over broth, provided no more than a trace of fermentable carbohydrate, which would inhibit indole production,

tube is cautiously removed to avoid soiling the upper part and the cotton plug firmly replaced.

The amount of medium added to each tube depends on the nature of the medium and the purpose for which it is to be used.

It is customary to introduce about 10 ml. of broth or peptone water into each $6 \times \frac{1}{2}$ inch tube but, for most purposes, a smaller volume in a narrower tube would be quite adequate.

Since some of the carbohydrates used in the so-called sugar media are expensive, narrow tubes ($\frac{3}{8}$ inch) are commonly used. With them, 3 ml. of medium per tube is sufficient. An exception to this must be made in the case of lactose bile salt broth used in the examination of water, as large volumes of this are required in tubes or even in bottles to receive the correspondingly large volumes of water tested.

Sterile solutions (saline, citrate etc.) which are cheap are commonly tubed in $6 \times \frac{1}{2}$ inch tubes each containing about 10 ml.

✓ "Slopes" or "slants" are prepared by sterilising about 5 ml. of agar or other solid medium in $6 \times \frac{1}{2}$ inch tubes. After sterilisation, the tubes are arranged on a flat bench on which rests a bar of wood or a glass tube about 2 cm. in thickness. The upper part of the tube rests on the bar, the bottom on the bench itself. The medium becomes solid in this position but it is advisable to leave the tubes undisturbed for about 24 hours when they can be stored upright. The agar is then found to form a smooth layer reaching from the bottom about one-third of the way up the tube.

✓ "Stabs" are tubes containing from 15 to 20 ml. of agar or gelatin. They derive their name from their use in making a culture by stabbing the medium from the top towards the bottom with a straight platinum wire inoculated with material containing

agar, a larger amount is recommended. When more than a few plates are required at a time, it is more convenient to store the agar in bulk—90 ml. for 5 plates, or 180 ml. for 10 plates—in flasks or bottles of suitable size.

The addition of gentian violet (1 : 500,000) to blood agar greatly facilitates the isolation of streptococci, as these organisms grow freely on this medium which inhibits the growth of almost all other Gram positive bacteria.

growth of the majority of Gram positive bacteria but not of those which are Gram negative.

Nutrient Gelatin

Leaf gelatin is dissolved in broth with the aid of heat. After adjustment of its reaction, the medium is clarified by treatment with egg white and filtration. It must be sterilised by the inter-

This medium is now practically never used in plates for the isolation of pure cultures of bacteria, but almost entirely in tubes (stabs) for ascertaining if the organism inoculated into it liquefies the medium.

Media Containing Indicators

tubes coloured differently or by inserting in the tube a coloured glass bead, but unfortunately different laboratories have different colour conventions. The basis of the "sugar" media is peptone water. To this is added the carbohydrate and an indicator to show any change of reaction which may occur as the result of bacterial growth. Litmus and neutral red are frequently employed,

Nutrient Agar

Agar fibre or powder is dissolved in broth in a steamer. After the reaction has been adjusted, the medium is steamed, filtered and sterilised in the autoclave. This medium requires a temperature of almost 100° to liquefy it but, when cooled, does not set until the temperature has fallen to about 43° . It is not liquefied by the action of any of the pathogenic bacteria.

Nutrient agar is a fairly stiff medium. A modification, known as "sloppy agar", is made by adding 1 volume of melted nutrient agar to 6 volumes of hot broth and cooling the mixture. This is a semi-solid medium very useful for isolating motile forms of bacteria which, unlike non-motile organisms, can migrate through it.

Strictly speaking the term "agar" means the jelling material added to broth to make nutrient agar but, in the everyday, "d nutrient

medium, by the addition to it of glucose (0.5 per cent.) or of citrated blood, serum or hydrocele or aseptic fluid (5 to 10 per cent.). These should be added to the melted agar and thoroughly incorporated in it before the medium is allowed to cool in sloped tubes or is poured into Petri dishes. In the case of blood or other body fluid which would be altered by heating, the agar must be cooled to about 55° before the addition is made.

For a type of blood agar known as "heated blood agar", "boiled blood agar" or "chocolate agar", the mixture of agar and blood is deliberately heated. From 5 to 10 per cent. of citrated blood is added to melted agar at 100° and the tube or bottle is placed in a boiling water bath where it is left for 1 minute after which it may be cooled and used for the preparation of slopes or plates.

By the addition of various chemical substances, agar or blood agar may be transformed into selective media, permitting the growth of wanted types of bacteria and preventing the growth of others.

sometimes employed in examining the fermentative capacity of bacteria. The tubes are inoculated when the medium is fluid, and this is then allowed to set. After incubation, acid production is shown by a change in the colour of the indicator, and evolution of gas by the breaking up of the medium or the presence in it of bubbles of gas. When the amount of gas formed is small, it may be more readily detected in this than in a fluid medium in Durham tubes.

With certain organisms (e.g. pneumococci, streptococci, neisseriae, diphtheria bacilli) the broth or peptone water carbohydrate medium does not give very good results, owing to the poor growth obtained. It may, however, be rendered suitable by the addition, to each tube of the sterilised medium, of one-fifth its volume of sterile hydrocele or ascitic fluid or serum. Such tubes should be incubated for 24 hours at 37° prior to inoculation in order to detect any which have become contaminated.

in the steamer.

Milk, freed from cream, and with the addition of an indicator It should be tubed and sterilised by steaming.

Media for the Culture of *C. diphtheriae*

L.H.C.
M

1. Coagulated Serum Medium

A large sterile vessel, such as an enamelled pail fitted with a lid, is two-thirds filled with the blood of a horse or ox, the blood being collected in a slaughter house. While absolute sterility is unattainable, every effort should be made to prevent avoidable contamination. The serum which separates from the clot is aspirated or pipetted off After standing for 24 hours in the cold to allow the

but we have found Andrade's indicator much superior to these. The reaction of colour. It is the bottom of which when the fluid is added, float owing to the air enclosed in them. in the steamer by the intervention, as high temperatures are As a result of the steaming

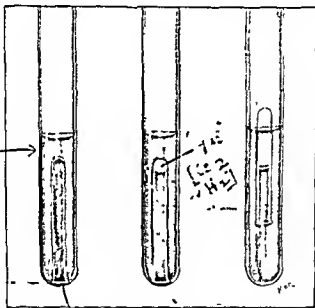


FIG. 12.—THREE DURHAM TUBES, SHOWING NO GAS, A SMALL BUBBLE, AND TUBE FLOATING DUE TO CONSIDERABLE GAS FORMATION.

the air is expelled from the inner tubes and the fluid fills them completely. A bacterium grown in one of these media may not have any effect on the carbohydrate, or, it may produce an acid or gas. The production of gas is indicated by the evolution of gas, which causes the collection of a bubble at the top of the medium (agar) containing the carbohydrate.

2) medium (agar) containing the carbohydr

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detect any which have become contaminated.

in the steamer.

Milk, freed from cream, and with the addition of an indicator such as litmus solution, is occasionally useful in the identification

Media for the Culture of *C. diphtheriae*

*Löffler's
Medium*

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A large sterile vessel, such as an enamelled pail fitted with a lid, is two-thirds filled with the blood of a horse or ox, the blood being collected in a slaughter house. While absolute sterility is unattainable, every effort should be made to prevent avoidable contamination. The serum which separates from the clot is aspirated or pipetted off. After standing for 24 hours in the cold to allow the

cells to deposit, it is sterilised by filtration through a Seitz filter using an EK (GS) disc. Then the serum alone or, preferably, a 3 : 1 mixture of serum and broth containing 1 per cent. of glucose, is filled into sterile tubes, or 1 oz. round screw cap bottles. These are placed in an inspissator which is a water-jacketed chamber heated by electricity or gas. Tubes rest on a rack which holds them in a sloped position, so that the fluid spreads about one-third of the way up their sides. Bottles are held in a horizontal position. The inspissator is gradually heated to 85° and kept at that temperature for 2 hours. The serum will then have set into a white or light yellow coloured solid of firm consistency. This medium is commonly sterilised in the steamer by the intermittent method. If the serum is sterilised by filtration and is filled with proper precautions into sterile tubes, this final sterilisation is unnecessary and should be omitted as it renders the medium much less satisfactory. This is usually, although incorrectly, termed "Löffler's medium".

2. Selective Media for *C. diphtheriae*

A considerable number of special media are used to facilitate the isolation of *C. diphtheriae*. The basis of all these is nutrient

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ria bacillus

assume a morphology so different from that of the same organism

grown on serum as to render microscopic identification difficult or impossible without subculturing on serum. Most of these disadvantages can be overcome by avoiding excessive heating of the medium both before and after the addition of blood and by keeping the concentration of tellurite low.

Media for the Isolation of Pathogenic Intestinal Bacilli

Very many media are used to facilitate the isolation of intestinal pathogens from materials, such as faeces, which contain coliform bacilli. These may be divided into those which are differential and

latter, which may be either solid or fluid, more or less completely suppress the growth of coliform bacilli.

We give the formula of only one differential medium, that of MacConkey, because we have not found any of the newer differential media superior to it.

1. MacConkey's Medium

MacConkey's medium contains peptone, sodium taurocholate, lactose, neutral red and agar. Peptone is the main nutritive material. The presence of sodium taurocholate renders the medium to some extent selective in that it tends to suppress the growth of non-intestinal bacteria. The presence of lactose and an indicator confers on the medium its differential properties.

Organisms of the coliform group ferment this sugar producing

The following are, in our experience, two of the most useful solid selective media for intestinal organisms. They are called selective because they completely suppress the growth of Bact

coli and also, more or less completely, of other organisms of the coliform group, while permitting the free growth of some or all of the intestinal pathogens. It would be quite wrong to assume that any organism growing on these media is a pathogen since many bacteria, such as those of the genus Proteus, frequently present in faeces, grow on them, but any organism found in culture on these media should be considered as a possible pathogen until it is proved not to be one.

2. Desoxycholate-citrate Agar *(Pale Pink color)*

This is Leifson's medium modified by Hynes. In addition to the nutritive materials supplied by meat extract and peptone, this medium contains lactose and neutral red, serving the same purpose as they do in MacConkey's medium, and sodium citrate, sodium thiosulphate, ferric citrate and sodium desoxycholate.

alteration of the neutral red by the acid produced by the fermentation of lactose. *Proteus* grows well on this medium but does not

tend to suppress the growth of rough variants of *Shigella sonnei*. Colonies on this medium should not be used for slide agglutination.

3. Wilson and Blair's Medium (*Pale green medium*)

In addition to nutrient agar, this medium contains bismuth-ammonium-citrate, sodium sulphite, sodium phosphate, glucose, ferric citrate and brilliant green, all of which appear to be essen-

th.
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B
Proteus is not.

The medium should not be kept in plates for more than 24 hours before it is used. It is of a pale green colour. Colonies of *Salmonella typhi* and *Salmonella paratyphi B.*, when close together and therefore small, are green but, when well dispersed, are black and the medium in their vicinity is also blackened and shows a

bacteria, the two solid selective media may be much more thickly spread with faeces than should a differential medium such as desoxycholate-citrate medium or a black colony on Wilson and Blair's medium might, at first, be assumed to be a pure culture. But a loopful of it transferred to a fluid medium such as lactose-peptone water may contain, among millions of typhoid bacilli, one or two coliform bacilli. These would grow and ferment the lactose, producing acid and gas in the medium, a result which would probably lead to abandoning further investigation of the bacteria in the colony. Colonies from these selective media should never be sub-cultured directly to fluid media. The correct procedure is to replate on a differential medium, such as that of MacConkey. On this the many colonies of the non-lactose fermenter can easily be distinguished from the few colonies of the lactose fermenter, and an indubitably pure culture obtained for further investigation.

✓ A further advantage of replating is that both the flagellar and

Thermophilic antigens of salmonellæ may be insoluble when the

Despite the fact that these two solid selective media can be fairly heavily inoculated with faeces, it is often an advantage to examine a larger amount of faeces than can be spread on a plate as, when pathogens are very few, the probability of detecting their presence is increased by increasing the amount of faeces examined. This can be done most satisfactorily by using fluid selective media, sometimes called enrichment media, two of which are described.

4. Tetrathionate Broth

This medium contains broth, chalk, sodium thiosulphate, iodine and potassium iodide. It should be used within a few days of its preparation. It is excellent for the isolation of the majority of the salmonellæ, but not for the shigellæ. It permits fairly rapid multiplication of the salmonellæ, even when the number introduced into a tube is small, and either completely suppresses or considerably retards the multiplication of coliform bacilli. Unfortunately it does not seriously impede the growth of Proteus, an important disadvantage in tropical countries where organisms of this genus occur more commonly than in temperate climates. A tube of this medium should be heavily inoculated with faeces. After over-night incubation, a loopful should be plated. Commonly MacConkey's medium is used and this has the advantage of enabling lactose and non-lactose fermenting bacteria to be distinguished. If only one plate is to be used, it is, in our opinion, best to use a plate of desoxycholate-citrate agar which facilitates the isolation of pathogens even when their proportion has not been greatly increased by culture in the fluid medium. Where economy of time and material is not essential, it is recommended that a loopful of the tetrathionate broth culture be plated on each of the media.

5. Selenite Medium

This medium, which contains sodium acid selenite, peptone, lactose and sodium phosphate, is particularly useful for the isolation of organisms of the enteric group. It is also of value for other salmonellæ but is useless for shigellæ. It can be stored in the refrigerator for several months. It should be heavily inoculated with faeces (1 g. per tube) and incubated over night. Isolation is carried out as described above for tetrathionate broth.

Media for *Myco. tuberculosis*

1. Griffith's Egg Medium

This is a plain egg medium, that is it contains nothing but egg and water, coagulated by heat. It is used mainly for the cultivation of the tubercle bacillus. It may be modified by the addition of sufficient basic fuchsin to give the finished medium a pale pink colour, so rendering growth on its surface more easily seen. A further modification is the addition of 5 per cent. of glycerol. To avoid drying of the medium during the prolonged incubation required for the culture of the tubercle bacillus, rubber caps should be tightly tied over the cotton plugs of tubes or the plugs should be treated with melted paraffin wax. The use of small screw cap bottles to contain the sloped medium is strongly recommended.

2. Lowenstein-Jensen Medium

In addition to egg, this medium contains potato starch, asparagin, glycerol and minor constituents. It is coagulated by heat in the same way as Griffith's medium and the same precautions must be taken to prevent drying of the medium. This medium is particularly useful for the primary growth of the tubercle bacillus, colonies developing more quickly on it than on plain or glycerol egg media.

3. Dubos Medium

This is a fluid medium in which, when heavily inoculated, tubercle bacilli grow rapidly and diffusely in the depth of the

medium in distinction to the pellicle type of growth which occurs in the usual fluid media used for the culture of the organism. The constituent of Dubos medium which is responsible for the type of growth is a water soluble synthetic ester of oleic acid, known as Tween 80.

Media for Anaerobes

As will be explained in a later chapter, anaerobic bacteria may be grown in or on the surface of ordinary media in a jar from which all oxygen has been removed. It is often convenient, however, to be able to cultivate anaerobic bacteria without the use of special apparatus to secure anaerobic conditions. This may be done by the use of special fluid media in which the presence of reducing substances keeps the oxygen tension at a sufficiently low level. Such media are more efficient in securing anaerobic conditions if they are used in narrow tubes, half filled, and if these are immersed for a few minutes in boiling water to expel dissolved oxygen and are then rapidly cooled before inoculation. Difficulties occasionally arise from the transfer of oxygen from the surface to deeper parts by convection currents. These may be overcome in either of two ways—by covering the surface with a layer of liquid paraffin or vaseline which prevents or slows the absorption of oxygen by the medium or by the incorporation of a small amount (0.05 per cent.) of agar in the medium which prevents the formation of currents. The first method is commonly used in connection with Robertson's medium, the second with Brewer's and other media to which a soluble reducing substance has been added.

1. Robertson's Minced Meat Medium

This medium consists of minced meat (10 g.) covered with 0.5 per cent. glucose medium. The top of the broth may be covered with a layer of liquid paraffin or melted vaseline. Capillary pipettes must be used to inoculate and to withdraw samples from the medium when an oil or vaseline seal is used. Robertson's medium is very useful for preserving cultures of certain organisms such as *Str.*

pyogenes, which are facultative but not obligatory anaerobes and which die in a short time under aerobic conditions.

2. Brewer's Medium

The addition of 0.1 per cent. sodium thioglycollate to broth renders it capable of supporting the growth of strict anaerobes. The medium is improved by the further addition of glucose. Very commonly 0.05 per cent. of powdered agar and 1 : 500,000 of methylene blue are added to the medium. Other substances including cysteine hydrochloride (0.2 per cent.) and ascorbic acid (0.1 per cent.) act in the same way as sodium thioglycollate and can be substituted for it.

3. Reduced Iron Medium

The simplest of the anaerobic media is made by adding to a tube of ordinary or glucose broth, freshly boiled and cooled in a narrow tube, a strip (3 × 5 mm.) of sheet iron, gauge 26, or an iron nail, sterilised in the hot air oven or Bunsen flame.

Media for Distinguishing Coliform Bacilli, *Insert in*

1. Buffered Glucose Broth

This is glucose peptone water, buffered with potassium phosphate. It is used in tubes for the Voges-Proskauer and methyl-red tests. A 24- or 48-hour culture is most suitable for the former. A 4-day culture must be used for the methyl-red test, since a

2. Koser's Citrate Medium

An ammonium salt is the source of carbon in this medium. aerogenes, grow in it and Growth is judged by the development of turbidity. It is essential to inoculate the medium lightly—one loopful of a broth culture is adequate. If too large inocula are used, these may add sufficient

carbohydrate or amino acids to permit growth of organisms otherwise incapable of growing in the medium.

Sterile Solutions

It is a great convenience always to have available in the laboratory a supply of sterile solutions including distilled water, physiological saline, carbol saline, citrate and glucose. These should be autoclaved in tubes or screw-top bottles. Labelling is avoided if each is distinguished by a particular colour displayed by tinting the cotton plugs, by painting the screw tops or by including a bead of appropriate colour in the tube or bottle.

Blood

Blood is a valuable addition to broth or agar, not only because it supplies desirable nutrients, but also because its red cells, which remain intact in the medium, enable one to detect if an organism produces a hæmolyisin which lyses the cells.

For laboratories where only a small amount of blood is required, this may be obtained from human volunteers by vein puncture or from rabbits by cardiac puncture. In either case, full aseptic precautions must be taken by sterilising the syringe and needle and by treating the skin with iodine before it is punctured. The horse is the most convenient source of larger quantities of blood and, for many purposes, its blood is preferable to that of other animals. Blood, whatever its source, should be collected in tubes or bottles containing sterile citrate solution. One part of this solution is sufficient to prevent the clotting of ten parts of blood, provided the solution is evenly diffused throughout the blood. Citrated blood may be kept without deterioration for 10 days or longer if stored in a refrigerator. The temperature should not be allowed to fall below 0°. The cells which, during storage, sink to the bottom of the vessel, should be dispersed before the blood is used.

Serum is obtained by collecting blood in a dry sterile vessel and allowing it to clot. When the clot contracts, serum is expressed. The cells deposit and the clear serum may be removed with a pipette.

CHAPTER V

THE MAKING OF CULTURES

The microscopic examination of bacteria enables them to be grouped into certain large classes on morphological grounds, but by this method scarcely any organism can be definitely identified. One of the most essential steps in bacteriology is to secure a pure culture of an organism. A pure culture is one in which only a single type of organism is present, it is usually the offspring of a single organism. Before describing the methods used for attaining this end, it is necessary to give some account of the apparatus used in the cultivation and examination of bacteria.

Certain pieces of apparatus, similar to those employed in chemical, zoological, and botanical laboratories, are essential for the study of bacteria. Of these the most important are test tubes, Petri dishes, commonly called plates (which name is a relic of the days when the pioneers in bacteriology made cultures on gelatin medium poured on flat glass plates), flasks, pipettes, graduated cylinders and thermometers. An electrically driven centrifuge is a necessity. One of the most efficient and reasonably priced of these is the angle centrifuge. Every centrifuge should have a free-wheel device to prevent the tubes being brought to rest too rapidly when power is cut off, as this disturbs any deposit formed. It is essential to balance the centrifuge by placing in the opposite bucket a tube similar to the one used and containing the same quantity of fluid.

Some form of suction apparatus, of which a good type is a Geissler's vacuum pump, is very useful. This can be used in filtering and also for withdrawing the supernatant fluid after centrifuging so as to leave the deposit undisturbed.

Probably the most used piece of apparatus is the platinum wire. Several pieces of this wire (or of nichrome, a fairly good substitute)

of different thickness and about 6 cm. long are required. These are mounted either in aluminium holders or glass rods. Some should be straight and others should have their free ends bent into



FIG 13 —ANGLE CENTRIFUGE
(Messrs Baird and Tatlock
(London), Ltd)

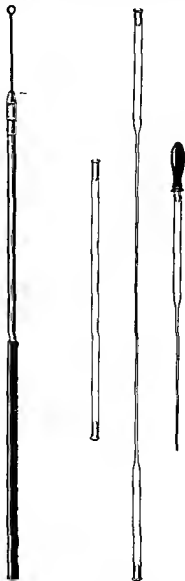


FIG 14 —PLATINUM
WIRE IN ALUMINIUM
HOLDER ($\times \frac{1}{2}$)

FIG 15 —THE
PREPARATION OF A
PASTEUR PIPETTE.

loops. This can be done by moulding the end round a glass rod which has been drawn out in the flame. The loop should be about 4 mm. in diameter, and must be closed so that it can be used for transferring drops of fluid.

Another necessity is a supply of glass tubing about 30 cm. long and 0.7 cm. in external diameter. Both ends are rounded in the flame, plugged with cotton wool, and the tubes are sterilised in the oven. When a pipette is required for the transference in a sterile manner of a larger quantity of fluid than that held by a platinum loop, one of the prepared tubes is held, one end in each hand, and the central part heated in a Bunsen flame, the tube being rotated all the time. When thoroughly soft, the glass walls fall in slightly and thicken, and then the tube is removed from the flame and the ends drawn slowly apart, the tube being rotated during this operation. When of sufficient length, the centre is again heated and the ends sharply pulled away from one another. This will produce a very fine tube, which is easily broken, and the ends can be sealed in the flame, so protecting the two pipettes thus produced until they are required. A rubber teat is fitted to the plugged end (without removing the cotton) and the capillary portion broken at a convenient length. A well-made Pasteur or capillary pipette of this type should have a length of about 12 cm. unnnarrowed, a rapidly tapering part of about 1 cm. and a very slowly tapering portion of about 10 cm., of which the proximal part will have an external diameter of about 2 mm. and the distal of about 1 mm. If the drawn-out part tapers too sharply, either the tube has been insufficiently heated or the ends have been pulled apart too rapidly. A considerable number of tubes should be prepared at one time and a pair of pipettes made from one of them when required.

Spreaders, the use of which will shortly be described, can be made from thin glass-rod, but those made from an iron wire are more permanent and, if kept polished, are quite satisfactory.

When it is desired to transfer bacteria from one culture tube to another, that is, to make a sub-culture, the following is the procedure. The plugs of all the tubes used should first be passed

by rapidly
which are on
the culture.

The plugs are then twisted in the tubes to break down any adhesions. The two tubes are held in the left hand, the exact method of holding them being unimportant so long as the medium is not obscured by the hand. Some hold one tube between the thumb and forefinger, the other between the fore and middle fingers, in each case the upper part, about 5 cm. from the top, being grasped. Personally, we prefer to hold the extreme bottoms of the tubes between the tip of the thumb and the bunched tips of the fingers. In this way three or four tubes may be held at the same time radiating like the ribs of a fan. The projecting portions of the plugs are grasped between the fingers of the right hand near their



FIG. 16.—SPREADER.

webs, the interior part of the plugs being directed away from the palm. If necessary two, or even three, plugs may be held between two fingers. After removing the plugs, the mouths of the tubes are quickly passed through the flame to burn off any adherent cotton. The holder of the platinum loop is held like a pen in the right hand and the wire heated to redness, when the lower part of the handle is passed a few times through the flame. The sterilised wire is then introduced into the tube containing the culture. The wire may be cooled, either in the water of condensation in the tube or by touching a part of the medium on which there is no growth. The culture is then touched with the loop, to which some will adhere. The charged loop is withdrawn and introduced into the other tube, care being taken to prevent it touching the mouths or sides of the tubes. The medium is inoculated, the wire is sterilised in the flame, and the plugs replaced, each to its own tube. Throughout the whole operation the tubes should be held as

nearly in a horizontal position as the medium permits to prevent the entry of bacteria falling from the operator or those floating in the air.

In all operations in which the use of a platinum wire is involved, it is essential to sterilise it twice, once before the material containing the bacteria is touched to prevent the culture being contaminated, and the second time after the culture has been made and before the wire is laid down, to prevent accidental infections. When the loop contains a mass of bacteria or a drop of infected fluid it should first be held some distance above the flame and then gradually lowered, as sudden heating frequently causes spurting, which may lead to contamination of the bench.

✓ Sloped or slanted media are inoculated by drawing the charged loop gently over the surface from below upwards in a straight line or, if the maximum amount of culture is desired, by rubbing the whole of the surface with the loop. ✓ Stabs of agar or gelatin are inoculated with the straight wire which, after it is charged, is pushed gently straight down into the medium in the middle line. ✓ In the case of gelatin, if it has been prepared a considerable time before this, a crack is likely to be caused which may interfere with the typical growth characteristics. The remedy is to melt the medium and allow it to set again a short time before it is inoculated. ✓ Fluid media are inoculated either by gently shaking the loop in the medium or by emulsifying the material in the fluid on the side of the tube just above the level of the main bulk of the medium and then washing this off by slanting the tube.

✓ Since several varieties of bacteria frequently occur together in nature, it is essential to learn the methods of obtaining pure cultures from material containing two or more varieties of bacteria. With the use of fluid media this can be done only in exceptional cases, and both luck and perseverance are necessary.

Solid media allow us to isolate an organism, even when it is

the density of bacteria
until each organism
being in the depth.

bacteria, while able to multiply, are unable to move and so the masses of bacteria resulting from the multiplication of each are well isolated. These masses, which are sufficiently large to be easily visible with the naked eye, are called colonies. A colony is usually a pure culture, that is consists of only one type of bacterium. If the colonies, whether on the surface or in the depth of the medium, are well separated, it is possible to touch one with a sterile wire and to transfer a portion of it to a tube of medium which, after incubation, will contain a pure culture.

Tubes are made in the depth
of bacteria in the material:

make several plates. Three agar stabs are placed in a vessel containing water, which is boiled for a few minutes until the agar is melted. The water is cooled by the addition of cold water until its temperature is about 50°.

A loopful of the material is transferred to one of the tubes with the usual precautions, and the whole thoroughly mixed by rotating the tube gently between the hands without allowing air-bubbles to form. Five loopfuls of the contents of this tube are transferred to the second, and after the same treatment a similar amount from the second to the third tube. The contents of each tube are poured into sterile Petri dishes (plates) and, after solidification, the plates are incubated. If the material contains a very large number of bacteria the first plate will probably show far too many colonies, but either the second or third ought to give a

plates should not be removed from the paper or box in which they were sterilised until the medium is ready. They should then be placed on a horizontal table in a place free from dust. The outside of the tube is dried, the plug is removed, the mouth flamed, and the lid of the plate is lifted at one side just sufficiently to allow

the tube to be introduced beneath it and the medium is poured into the plate. The lid is then replaced, and the plate is not moved until the medium is thoroughly set. At no time, either before or after the medium is poured into the plate, is its interior left un-

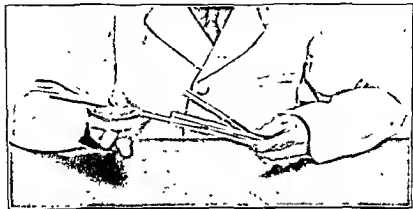


FIG 17—THE METHOD USED TO INOCULATE A TUBE OF MEDIUM
(Note how the tubes, platinum wire and plugs are held)

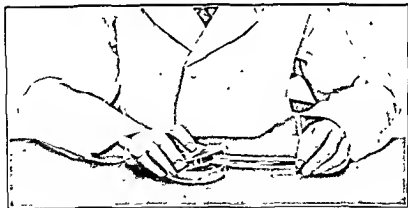


FIG 18—POURING A PLATE
(Note how the lid is held over the plate)

covered by the lid, as this may permit the entry of bacteria from the air. The procedure of plating as described above requires

some care and judgment, as it is necessary to avoid over-heating the bacteria, which might kill them, and also to guard against the medium solidifying before the plate is poured. Agar melts slightly below 100° and sets at 42° to 44° . Since the medium was at 50° before the infected material was added the work must be done rapidly and the plate poured before its temperature has fallen to 44° . Where gelatin is used the technique is simpler. The medium may be inoculated at a temperature of about 35° , and a considerable time elapses before it sets at air temperature. Gelatin cultures are incubated at 20° to 22° .

✓ Agar plates are always incubated in the inverted position, i.e. lid downwards. If reversed, the water of condensation would collect on the lower surface of the lid and fall on the medium. Some of the bacteria on or in the medium might grow in this water and spread over the surface, rendering the culture useless.

✓ Gelatin plates are, however, incubated with the lid uppermost as, if inverted, any liquefaction of the medium by the growth of liquefying organisms would destroy the culture.

For spread or streak plates the method differs somewhat from the above. The medium is melted and cooled as before and poured into plates. It is always advisable to cool the medium before pouring it, for thus the plate is less likely to be cracked, and also the amount of condensation water on the lid will be less. ✓ Plates should not be used for spreading until at least an hour or two after pouring, as then the surface will be both firmer and drier. ✓ With a wet plate there is always the risk of an organism multiplying in the fluid and spreading over the entire surface.

with active bacteria. ✓ For this reason,

for isolating pathogenic intestinal bacteria (as MacConkey's medium) must be dried in the incubator before use.

For the routine plating of pus and other morbid fluids from the body, blood agar is preferable to plain agar. An agar stab is melted and cooled to 55° . About 1.5 ml. of citrated blood, collected as described in Chapter IV, is added by means of a

sterile Pasteur pipette and thoroughly incorporated with the agar, either by rolling the tube between the hands or by pouring into another sterile tube and back again. It is then poured into the plate and allowed to set. A properly made blood agar plate should be uniformly translucent and of a moderately deep red colour. A simpler alternative is to place the necessary amount of blood in the plate and pour on it the melted and cooled agar. The covered plate is then lifted in both hands and rocked so as to diffuse the blood throughout the medium. This operation must be completed before setting commences, as otherwise an uneven surface will result. This method never gives such a uniform mixture as that described above, but the risk of accidental contamination is less and the plates are quite satisfactory for routine use. Plates of agar enriched with serum, hydrocele or ascitic fluid are made in exactly the same way. Glucose-agar may be prepared by adding one-tenth volume of a sterile 10 per cent. solution of glucose to the melted agar before pouring

Plates intended for spread or streak cultures should always be used on the day on which they are made. In pouring the plate there is the possibility of a bacterium falling on the surface of the medium. If the plate is used fresh, a single contaminating colony will result, and this can generally be distinguished without difficulty from the colonies due to the bacteria in the material under investigation. If, however, the plate has been kept from the previous day, the bacterium may have produced a colony too minute to be seen with the naked eye or hand lens, but nevertheless consisting of thousands of bacteria. In the spreading operation these bacteria are distributed over the surface of the plate, where they give rise, on subsequent incubation, to a very large number of colonies which obscure those of the bacterium in the inoculum.

Two methods are used for inoculating the prepared plates. One employs a wire loop, the other a glass or metal spreader. In the first method the loop, charged with the material for culture, is drawn lightly over half the plate in a series of parallel lines (A1 to A9) about 0.5 cm. apart. The plate is then rotated through

90°, the loop sterilised and another series of streaks (B1 to B9) made on half the plate at right angles to the first series, each streak starting in the previously inoculated half and finishing in the previously uninoculated third quadrant of the plate. The plate is then rotated a further 90°, the loop sterilised and a final series of streaks (C1 to C9) made from the third to the previously uninoculated fourth quadrant. It is important to sterilise the loop between each spreading. By this technique well separated

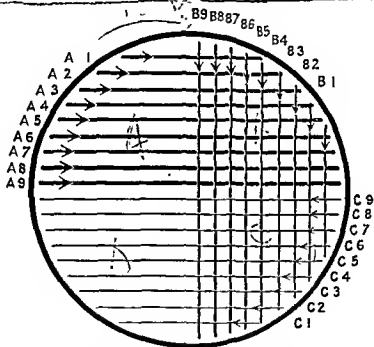


FIG 19.—SPREADING PLATE BY METHOD OF PARALLEL STREAKS.

colonies are usually found in one quadrant of the plate irrespective of the size of the inoculum.

When the spreader is used, a loopful of the material is placed near the edge of the plate and the spreader sterilised in the flame and allowed to cool. Before it is used it should touch the water of condensation on the lid to make quite certain that it is not too hot. With the spreader the drop is well rubbed over a small portion of the surface, then the spreader is lifted and rubbed over a fresh

area. This is repeated until the whole of the surface has been rubbed. A good plate—that is one showing on some part well-defined if about seven or eight areas never being returned to an area better results are obtained by increasing the area devoted to each rubbing and using two plates instead of one. During spreading by either method, the plate



FIG. 1. BACTERIAL CULTURE BY THE METHOD OF PARALLEL

contamination by holding the lid above it in the left hand.

The number of colonies depends on the number of organisms in the culture.

24

The plate must be

and a hand lens, both by reflected and transmitted light, and the different varieties of colonies noted. The lid is removed and the plate held in the left hand with its surface vertical. In the right hand is held the platinum wire, straight for minute colonies or with a loop for those of larger size. The wire is sterilised, cooled by touching a part of the medium devoid of growth, and the colony touched with it. The plate is restored to its lid and the



FIG. 21 —THE COLONIES ON A PLATE INOCULATED BY SPREADING.

wire rubbed over the surface of an agar slope or used for inoculating a tube of some other medium. This tube, after incubation, should contain a pure culture of the bacteria present in the colony picked. The operation may be facilitated by the use of a watchmaker's lens held in the eye. The picking of the deep colonies obtained when the material was mixed with the melted medium is similar, except that in this case the wire must penetrate the

medium to reach the colony required. In doing so it is necessary to avoid any colony lying above or below the one aimed at

In the majority of cases the use of one or other of these plating methods will see
ties of bacteria,
closely together

another plate, and so on.

symbiosis, that is the living together of two or more varieties of bacteria, the growth of one assisting the development of the other.

As has just been mentioned, this very commonly occurs with the

medium contains a carbohydrate fermentable by one, the acidity developed may inhibit the growth of the second; *L. acidophilus* in milk culture ultimately destroys the putrefying bacteria. *Ps*

in a mixture, a knowledge of the peculiarities of its structure or metabolism may suggest a method for its easy isolation, even when it is present in such small numbers as to make its separation by ordinary plating rather unlikely. As instances of this we may mention the use of blood-tellurite media for growing *C. diphtheriae* and the employment of Wilson and Blair's medium in detecting

the presence of enteric bacilli in faeces. The cholera vibrio grows well in a very alkaline medium and is strictly aerobic, and so we add faeces, suspected to contain the vibrio, to alkaline peptone water and, after incubation, we find on the surface an almost pure culture of this organism. Streptococci grow abundantly and quickly in gentian-violet glucose broth and form a deposit in the bottom of the tube which, when plated, will usually permit of their isolation, even when the original material contained a very small number together with large numbers of other bacteria. Many more examples of the use of such more or less selective media to

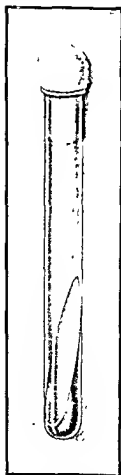


FIG 22—AGAR
(SLOPE IN TUBE.



FIG 23—AGAR SLOPE IN
SCREW CAP BOTTLE.

Selective heating, the latter can be killed, leaving the former still viable.

It is customary, when a colony is picked off a plate, to inoculate an agar or blood-agar slope with it, and so obtain a pure culture. When this culture has grown, sub-cultures may be made from it in media suitable for the identification of the organism, such as those containing various carbohydrates.

When cultures of very slowly growing organisms are made on sloped media in tubes, precautions must be taken to prevent drying, which

would interfere with growth. The plug may be cut off level with the top of the tube, which is covered with an india-rubber cap, or the plug may be immersed in melted paraffin wax. The use of small screw cap bottles containing the sloped medium is in every way more satisfactory.

All cultures should be fully labelled with the source, name of organism, and date on which the culture was made, as soon as the medium has been inoculated. Stick-on labels are unsatisfactory and dangerous, grease pencils or glass-writing ink are better.

The methods already described are applicable chiefly to the aerobic bacteria. For the cultivation of anaerobic bacteria, which grow only in the absence of oxygen, special procedures must be adopted. Either ordinary culture media in plates or tubes contained in a special apparatus, or media adapted for the growth of these organisms without any new apparatus may be used. The principles of the various methods are (1) exclusion of air; (2) exhaustion of air; (3) absorption of oxygen from the air; or (4) replacement of air by a neutral gas, such as hydrogen or nitrogen

gelatin, and inoculated, the added bacteria being diffused by rolling the tube without shaking, which would allow oxygen to be absorbed. It is then cooled and incubated in the ordinary way.

the colonies being picked after the removal of the cylinder of medium from the tube. This is done by cutting around the tube with a file or diamond at about the level of the middle of the column of medium. The exterior of the tube is sterilised with antiseptic solution, which is washed off with alcohol, the latter

being burned off. The tube is broken by applying a red hot wire or glass rod to the mark, when a crack will develop along the mark. The lower portion of the tube is removed and the cylinder of medium caught in a sterile Petri dish, where it can be examined and colonies picked.

Various special media for the cultivation of anaerobic bacteria in air have been described in Chapter IV. Now we must consider

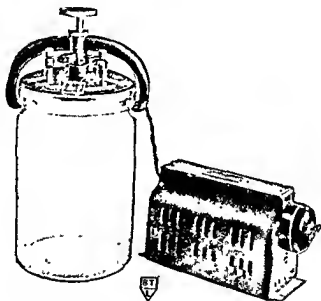


FIG 24—THE MCINTOSH AND FILDES ELECTRIC ANAEROBIC JAR
(Messrs Baird and Tatlock (London), Ltd.)

the special pieces of apparatus in which anaerobic bacteria may be grown in or on the surface of ordinary media.

The older types of anaerobic apparatus which relied chiefly on the replacement of air by hydrogen and the absorption of oxygen by alkaline pyro solution, have now been almost entirely superseded by the McIntosh and Fildes electrically heated anaerobic jar, which robs anaerobic methods of much of their difficulties. It consists of a glass or metal jar and a heavy metal lid which is fixed to the top of the jar by a clamp, an air-tight join being

effected with vaseline, plasticine, or a rubber washer. The lid is

gen is generated from zinc and sulphuric acid and purified by passing through wash bottles containing solutions of lead acetate silver nitrate and alkaline pyro to absorb H_2S , AsH_3 and oxygen). With both valves open, hydrogen is sent through the jar for some time until, when the gas escaping from the second valve is collected in an inverted test tube and ignited, it burns without exploding. The escape valve is then screwed down, the other being left open to admit hydrogen. The electrical terminals are connected to the supply through a suitable resistance. The current heats the

eliminate all oxygen contained within test tubes and plates) and is then switched off, the valve leading to the hydrogen supply being left open until the jar has cooled when it is closed, the hydrogen supply disconnected and the jar incubated. An indicator

3 ml, water to 100 ml, (c) glucose 6 gm., water to 100 ml and a crystal of thymol. The mixture, in a test tube, is boiled until colourless and put into the jar with the cultures. It remains colourless so long as oxygen is excluded, but turns blue in the presence of very slight traces of that gas. Plate cultures in the anaerobic jar are often damaged by excessive condensation of water. This may be prevented by putting in the jar the lid of a

Petri dish containing a layer of anhydrous, granular calcium chloride.

A small V-shaped piece of iron should be placed astride the rim of a plate before putting on the lid in order to allow the free exchange of gases between the culture and the atmosphere in the jar.

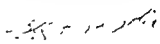
In dealing with anaerobes, the greatest difficulty is experienced in obtaining pure cultures. Ordinary plating methods, which usually succeed with aerobic organisms, frequently fail with the

uniform rate, neither outgrowing the other. It has frequently been found that a believed pure stock culture of an anaerobic bacterium, which may have been cultivated for years in a laboratory, really consists of two distinct species. This fact explains the great divergence which formerly existed, and even now exists although to a less extent, in the standard descriptions of the characteristics of certain anaerobes. Repeated plating, differential heating, by which less resistant bacteria or spores are destroyed, dilution methods and animal inoculation, if practised with great care and skill, may succeed in the production of a pure culture; but the only reliable method is by the isolation and culture of a single bacterium or spore actually seen with the microscope at the moment of its capture. The methods of doing this (such as that of Barber) are difficult and extremely tedious, and are impossible for the student, or indeed for any one who has not devoted a considerable time to mastering the details of the technique.

Certain bacteria grow best in an atmosphere of carbon dioxide; some grow only in a similar concentration: others, such as staphylococci, produce maximum amounts of toxin in the presence of about 20 per cent. of this gas. The growth of the majority of other, less fastidious bacteria, especially if the number inoculated is small, is improved by the addition of up to 5 per cent. of carbon dioxide to the

atmosphere in which they are incubated. For test tube or plate cultures of such bacteria, the necessary conditions are most easily provided in a jar, such as that of McIntosh and Fildes. The mixture of carbon dioxide and air or oxygen may be forced into the jar under pressure through one of the valves, so displacing the jar's original content of air through the other. Alternatively, carbon dioxide may be produced in the jar by adding acid to a tube containing a weighed amount of sodium bicarbonate immediately before the lid is closed (1 gm. NaHCO_3 produces 260 ml. of CO_2) or by placing in the jar a weighed amount of carbon dioxide snow (1 gm. yields 500 ml. of the gas).

For the cultivation of bacteria an incubator for maintaining the cultures at a constant temperature is essential. The optimum temperature of growth for the majority of human bacteria is that of the body, 37° . For gelatin medium a temperature of from 20° to 22° is suitable. Five water baths, adjusted to operate at 37° , 44° , 52° , 56° and 60° , are also frequently required for culture or other purposes.



CHAPTER VI

SPECIAL TECHNIQUE

In this chapter it is proposed to describe the technique of certain operations carried out in the laboratory.

Enumeration of Bacteria

The methods for estimating the number of bacteria in a fluid can be divided into two groups—microscopic methods and cultural methods. The former give an estimate of the total bacteria present, whether living or dead, the latter only of living bacteria.

It must be emphasised that no available method yields very accurate results and, even when every care is taken, errors of the order of + 25 per cent. must be considered quite moderate.

It is always worth while to devote considerable attention to the method of sampling so as to secure a representative sample and also to avoid contamination with material not under examination.

Before carrying out the examination, every effort should be made, by shaking or other mixing methods, to get as uniform a suspension of bacteria as possible since bacteria suspended in fluids have a marked tendency to adhere together in clumps.

Microscopic Methods

1. Probably the best microscopic method is that in which a special type of counting chamber, similar to a hæmacytometer with Thoma ruling but with the cell depth only 0.01 mm., is used. The suspension is accurately diluted with distilled or filtered water, the extent of the dilution (1 : 10, 1 : 100, 1 : 1000) depending on the probable number of bacteria present. A loopful of the well-mixed dilution is placed on the ruled area and the cover glass adjusted. The slide is left for a few minutes to allow the bacteria to settle and the preparation is examined microscopically, using the 1/6 inch objective and a high power ocular. The best results

are obtained if a dark ground condenser is used but, failing this, the diluting fluid should be tinted with a dye such as methyl violet. The number of bacteria seen in 100 small squares is counted and the average calculated. Since the dimensions of a small square are $1/20 \times 1/20$ and the depth of the chamber is $1/100$ mm, if the fluid undergoing examination was diluted $1/100$ and is 8, the fluid contains sub. mm.
 $= 32,000$ million per ml.

2. A much less accurate method is to lay a grease-free slide on a piece of paper ruled in square centimetres. A volume (usually 0.01 ml.) of the fluid is accurately measured on to the slide and, with a platinum wire, is spread evenly into a film covering exactly 2 sq. cm. The film is dried in air and is then fixed, the method of fixation depending on the nature of the fluid; if water or saline, heat fixation is satisfactory, but if milk, the film should be treated first with xylol in order to remove the fat and then with methyl alcohol in order to fix it on the slide. It is stained with a simple aqueous solution of methylene blue, gently washed with water and dried, preferably in the incubator. It should not be blotted as some of the film would be removed by the paper. Before the film is examined, the area of the field observed with the optical system of oil-immersion lens and ocular to be used is determined by using as object a glass scale divided into units each of 10μ . If the diameter of the field is 16 units ($= 160\mu = 0.016$ cm.) its area is 0.0002 sq. cm. The average number of bacteria per field is now determined. Since the area of the field is $1/10,000$ of the area of the whole film and since 0.01 ml. of fluid was spread in the film, the number of bacteria per ml. of the fluid will be 1,000,000 times the average number per field. This method, which is essentially that of Prescott and Breed, is occasionally used for the rapid examination of milk.

3. A method of estimating the number of bacteria suspended in water, saline or other clear fluid which, although not very accurate, is adequate for the standardisation of suspensions for vaccines, agglutination tests and many other purposes, is to compare the

opacity of the suspension with that of a standard tube which contains a suspension of barium sulphate. It is convenient to have a series of standard tubes of different opacities which were originally standardised by comparison with accurately counted

species per ml.

Cultural Methods

It is micro
(a) N

are dealing with bacteria as they occur in nature, in water or milk for example, no single medium or cultural method will permit the growth of all the organisms present. (c) Bacteria tend to occur in chains or clusters or to adhere together in clumps and, unless its component individuals, it will

s if it were a single individual.
y be used. The methods applicable to each will be considered in turn.

1. Agar is the solid medium most commonly employed. Into each of a series of sterile tubes, except the first, is measured 9 ml. of sterile diluting fluid. Either water or saline are commonly used, but certain types of bacteria die quickly in either and dilute broth (1 or 2 per cent. in saline) is preferable. Some of the fluid to be examined is placed in the first tube and, with a sterile 1 ml. pipette, 1 ml. is transferred from the first to the second tube: the pipette is returned to the first tube. With a fresh sterile pipette the contents of the second tube are thoroughly mixed and 1 ml. is transferred to the third tube: the pipette is returned to the second tube. This process is repeated until each tube, which now will have a pipette standing in it, contains a dilution (1 : 1, 1 : 10, 1 : 100 etc) of the fluid. The number of tubes used depends on the probable number of bacteria in the fluid. Agar stabs, corre-

sponding to the number of tubes, are heated in boiling water until the agar is melted. They are then cooled to 50° and to each is added 1 ml. of the contents of one of the tubes. This is incorporated in the medium by rolling the tube between the palms, and the inoculated medium is poured into a sterile Petri dish where it is allowed to set. The plate so formed is then incubated, usually for 24 or 48 hours, and most commonly at 37° but, for special purposes, a lower temperature (22°) or a higher (44°) may be used.

... id one is selected
 ... being as close to
 ... ly counted using

The number of colonies multiplied by the reciprocal of the dilution is capable of growing in agar. In stating the result only two figures are given. If, for example, 178 colonies are counted in the plate made with 10 ml. of the 1 : 1000 dilution, this should be reported as 180,000 bacteria capable of growing in agar per ml. This is often, rather inaccurately, shortened to 180,000 bacteria per ml. Both methods of reporting ignore the fact that a chain of streptococci or a clump of other bacteria gives rise to only one colony and so is recorded as only one bacterium.

(2) The information afforded by fluid media is limited. In one method, 1 ml. of the fluid to be examined and the same volume of each of a series of decimal dilutions of it are inoculated into tubes of broth which are incubated. Turbidity indicates growth and therefore the presence of at least one bacterium in the volume of fluid inoculated. If, in a series, the tubes containing 1 ml. of 1 : 1, 1 : 10 and 1 : 100 dilutions show growth and the tubes containing 1 ml. of 1 : 1000 and 1 : 10,000 do not, the result should be stated as—"bacteria capable of growing in broth were present in 0.01 ml. but not in 0.001 ml". This is frequently reported as "100 bacteria present per ml". Neither methods of reporting is accurate, but the second is much less accurate than the first. Irregularities exist owing to the impossibility of getting an absolutely even suspension and also, apart from this, they are inherent

in the method. Suppose a fluid containing 1000 bacteria per ml. is tested in this way, the quantity added to each tube being one-tenth of that added to the previous one. The tubes receiving 1 0, 0 1, 0 01 and 0 001 ml. should show growth and those with 0 0001 ml. and less should not. In practice, we might find that, in some tubes receiving 0 001 ml., the result would be positive and in others negative, and also we would occasionally find that, in later tubes, a few positives would occur because, although each one-thousandth of a millilitre should hold only one organism, this occupies only a very small part of that volume and would be present in a particular ten-thousandth or one-millionth part of a millilitre. It is advisable, if this method is used, to duplicate or triplicate each volume and to regard as positive only a volume which gives growth in the two or the three tubes inoculated with it as this reduces, to some extent, the errors of sampling. If each of three tubes of broth inoculated with 0 001 ml. of fluid shows growth, there is a much higher probability that the broth contains 1000 or more viable bacteria per ml. than if growth occurs in a single tube inoculated with the same amount. By increasing the number of tubes inoculated with the same volume of the fluid still greater certainty is achieved. For the examination of water it is now customary to inoculate one bottle with 50 ml. of water, five tubes each with 10 ml., and five tubes each with 1 ml. Statistical consideration of the possible results has yielded a set of tables known as McCrady's tables (see Chapter XLVIII). These show, for any combination of positive results, the "probable number of bacteria per 100 ml".

Broth has been described as the fluid culture medium used, but in practice broth is rarely employed. Much more commonly the medium is lactose bile salt broth and the method is used not to estimate the number of bacteria present but the number of lactose fermenting bacteria present. These, if present in water or milk, usually indicate faecal contamination.

For the purpose of estimating the number of other types of bacteria, media adapted to their cultivation may be used in a manner similar to that described for agar or broth.

Filtration

Filtration in the bacteriological sense is employed to obtain water or other fluids free from bacteria for domestic or laboratory use without resorting to heat. It is also used to separate soluble bacterial products present in a fluid culture from the bacteria themselves. The filters may

be composed either of un-glazed porcelain (Chamberland and Doulton), of diatomaceous earth (Berkefeld and Mandler), of sintered glass or of asbestos (Seitz). They are commonly made in the form of thick-walled tubes, called candles, closed at one end and with the other end fitted with some kind of attachment (metal in the case of the Berkefeld and glazed porcelain in the Chamberlain and Doulton filters), to which a rubber tube or stopper may be fitted. Since filtration without the application of pressure would be too slow to be practicable, it is

necessary to force the fluid through by applying pressure greater than that of the atmosphere or, as is more usually done, to utilise atmospheric pressure by exhausting the receiving vessel, which may be accomplished with a vacuum pump. Excessive pressure is to be avoided, as it may force bacteria through the pores of the filter and, on the other hand, filtration should be carried out as rapidly as possible, since bacteria may grow through even some of the finest filters if sufficient time is allowed. One method of using a small Chamberland filter is shown in Fig. 25. Here the fluid to be filtered is introduced into the candle, through the

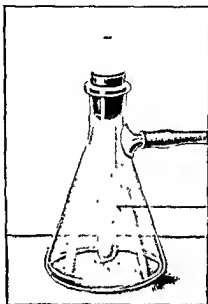


FIG 25—SMALL CHAMBERLAND FILTER.

opening in the top, by means of a pipette, and the filtrate is collected in the flask, filtration being effected from within outwards. In Fig. 26 a Berkefeld filter is shown arranged to filter from without inwards. The candle is surrounded by a glass mantle, open at the top, into which the fluid to be filtered is poured. In

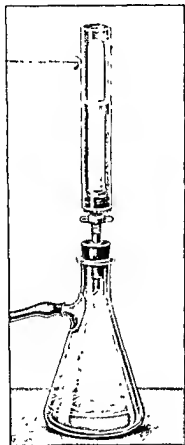


FIG. 26 —BERKEFELD FILTER

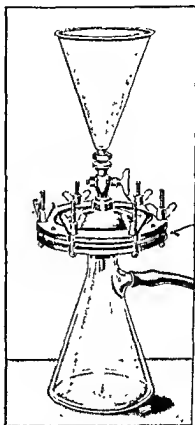


FIG. 27.—SEITZ FILTER.

both cases a negative pressure is created in the receiving flask by means of a vacuum pump.

Sintered glass filters are made of finely ground glass fused sufficiently to make the small particles adhere. A disc of this material is fused into a funnel, constituting a diaphragm, through

which fluid is aspirated by the negative pressure in the receiving flask.

The Seitz filter is made of metal and holds a disc of compressed asbestos through which fluid is obliged to pass in order to reach the receiving flask.

Different grades of filters may be obtained, the coarser of which (Chamberland L1 and L2, Berkefeld V and Seitz K (or FCB)) cannot be relied on to prevent the passage of bacteria. None of the ordinary bacteria will pass through Chamberland L3, L5, or F, Doulton, Berkefeld N or W, or Seitz EK (or GS) filters, and few of the filterable viruses will pass through Chamberland B, L7, L9, or L11 filters. The majority of sintered glass filters are merely clarifying filters but some are bacteria proof.

In all cases it is necessary to sterilise the assembled apparatus in the autoclave. When a candle has been used, its pores become choked and the filter works more slowly. The following method of cleaning can be recommended. The filter is soaked in bleaching lime solution to disinfect it. The outside is brushed with a stiff brush and is washed by aspirating or forcing saline through the wall in the reverse direction from that in which filtration proceeded. The candle is then boiled in 2 per cent. sodium carbonate solution for half an hour and then in several changes of water. Finally distilled water is again forced through the walls. All filters, whether new or cleaned, before being assembled should be tested for the presence of minute cracks. The simplest method is to immerse the candle under water and pump air into it by means of a rubber tube attached to it. If a crack exists, air will pass through it and the bubbles will be visible under the water. Sintered glass filters should be washed through in the reverse direction by water followed by nitric acid and this, in turn, by large amounts of water. Since a new disc is used in the Seitz filter for each filtration, the question of cleaning the actual filtering material does not arise.

Ultrafiltration is the term applied to filtration through collodion filters which prevent the transmission of all bacteria but allow smaller particles, such as viruses, to pass through. Collodion membranes are porous and, although the pores are always very

line, their size can be controlled by the exact method of preparation of the membranes. When the average pore size (A.P.S.) of one of these gallocol membranes, as they are called, is 10 $m\mu$, only the smallest of the viruses can pass through, while coarser membranes (A.P.S. 1 μ) permit the passage of almost all the viruses.

Animal Inoculation

Experiments on living animals are essential in bacteriology, but must not be performed in Great Britain or Ireland without a Government licence.

The common laboratory animals are guinea-pigs, rabbits, mice and rats. Material containing bacteria may be injected subcutaneously, intraperitoneally, intravenously or into the brain. Less commonly other routes, into the anterior chamber of the eye, the heart or lung, may be employed. Subcutaneous injections in guinea-pigs and rabbits are best made by shaving the abdominal skin, treating with tincture of iodine and inserting a fine needle obliquely into a pinched-up fold of skin. In the mouse or rat the skin near the root of the tail is most convenient. In intraperitoneal injection, after preparation of the skin of the abdomen, the whole thickness being pinched up between the finger and thumb, the needle is inserted into the midst of the fold thus formed, so avoiding penetration of the gut. When the fold is released the needle will be in the peritoneal cavity. In the rabbit the marginal vein of the ear is used for intravenous injection. The covering skin is shaved and the vein distended by light pressure on it near the root of the ear. A fine needle is then introduced and the injection slowly given. If a swelling forms, the fluid is in the subcutaneous tissues and not in the vein. Intravenous injection of the guinea-pig is difficult, but intracardial injection can be carried out quite easily. Injections can be made into one of the veins situated near the root of a rat's tail.

Blood samples may be required from the living animal. In the case of the guinea-pig, the animal is anaesthetised with ether (a cone made from cardboard to fit over the nose and mouth and containing cotton wool moistened with ether is an excellent mask)

and held extended on its back. A needle, similar to that used for human intravenous injections, is connected by a short rubber tube with a wide glass bulb to the other end of which a long rubber tube is connected. The apex beat of the heart is found and the needle inserted in a backward and inward direction. Suction is applied by holding the end of the rubber tube in the mouth, and blood usually appears in the glass tube or, if not, the needle may be slowly withdrawn, suction being maintained, as sometimes the needle penetrates the heart completely. In this way about 10 ml. of blood may be withdrawn without endangering the animal's life.

Blood may be obtained from a rabbit by cardiac puncture or, from its ear, by puncturing the marginal vein with a triangular needle and allowing the blood to drop into a tube. If the ear is kept warm and the skin previously rubbed with vaseline as much as 50 to 60 ml. of blood may be taken from a suitable rabbit. If a rabbit is to be bled to death, it is first anaesthetised and the blood collected from the carotid artery after the vessel has been exposed, or blood is aspirated from the heart as described above. A small blood sample may be obtained from the mouse or rat by cutting off the terminal portion of the tail.

An animal dying after inoculation should be thoroughly examined. It is tied on a board and the hair soaked with 2 per cent. lysol. The skin is completely reflected from the anterior of the thorax and abdomen; with fresh sterile instruments these cavities are opened and the organs required for culture are removed and placed in sterile Petri dishes. In many cases it is necessary to obtain a sample of the blood from the heart. This is done by searing the anterior surface of the organ with a cautery and penetrating this portion with a sharp sterile capillary pipette into which a few drops of blood are aspirated. Juice from other organs, for culture, may be obtained in a similar manner.

Antisera are widely used in the laboratory, agglutinating sera for the identification of pathogenic bacteria and hemolytic serum, which is used in the Wassermann Reaction, being those most commonly employed. For their production the rabbit is the animal of choice. Where very large amounts are required, how-

ever, horses or goats may be used. Rabbits may be injected subcutaneously or intraperitoneally, but the intravenous route is to be preferred. The skin over the marginal vein of the ear is shaved. By massaging the ear and then compressing it lightly at the base, the vein can be made to stand out prominently so that it can easily be entered by a sharp hypodermic needle attached to a syringe containing the inoculum. Since within wide limits antigenic response varies and since bacterial initial dose for the usually the largest death of the animal

reaction following the injection of a small number of the highly toxic salmonellæ or shigellæ, while fifty or more times the number of streptococci can be given without apparent effect, provided these have been washed free of exotoxin.

Three, four or more injections are given, the dose being increased on each occasion as immunity to the toxic constituents of the bacteria rises. As a rule, suspensions of killed bacteria are adequate. Occasionally, however, as in the case of the Vi antigen of *Salm. typhi*, maximum response is not achieved unless living organisms are used, but these should not be given until considerable immunity has developed from prior injection of killed cultures.

are those

Salmonel

the bodies (O) of the bacteria. To prepare an H antiserum, formalin is added (0.25 per cent.) to an actively motile broth culture of the organism which is then allowed to stand on the bench overnight to be sterilised. The killed culture is diluted with

0.25 per cent formalin solution

organism

when exa

culture are
must first be
ter bath for

2 hours. Treatment with alcohol, which destroys flagellar power to stimulate production diluted with saline to for H suspensions. One ml. constitutes the initial dose. A total of four injections, given at each occasion, animal is bled, to 7 days after the last injection. The blood is discharged into a wide tube or a bottle, which is left in a sloped position until a firm clot has formed. The serum which separates is pipetted off into a clean tube and cleared of residual red cells by centrifuging. Sera may be preserved by maintaining them in the frozen state or by the addition to them of an antiseptic such as chloroform or phenol. Phenol (2 per cent.) in glycerol (50 per cent) buffered at pH 6.5, is a valuable preservative for general use. One part of this is added to four parts of serum to give a final concentration of 0.4 per cent phenol and 10 per cent glycerol. The inclusion of the buffer enhances the keeping properties of the serum.

For the preparation of hemolytic sera, the red cells are thoroughly washed in saline and a suspension made one quarter the strength of the standard suspension used in performing the Wassermann reaction. The first injection consists of 1 ml. of this suspension. The subsequent steps in preparation are similar to those described for agglutinating sera.

Preparation and Use of Vaccines

Bacterial vaccines are suspensions of bacteria, living or dead, used for the production of active immunity. The organisms are

and heat (60°, or a somewhat lower temperature, for 1 hour). Since either phenol or heat or a combination of the two may destroy certain antigenic substances of some types of bacteria,

other methods of killing, such as by the use of alcohol, are sometimes employed. Samples of the suspension should be inoculated into fluid media under both aerobic and anaerobic conditions to determine both that the organisms used have been killed and that no living contaminant is present.

The number of bacteria in the suspension may be determined by any of the microscopic methods described for the enumeration of bacteria in this chapter. Since the action of vaccines on the patient is very much more variable than is that of drugs, we are doubtful if great refinements of counting methods are of much value.

Vaccines are diluted for use and are supplied either in sealed glass phials, each intended for a single dose, or in rubber stoppered bottles containing 10 ml. for repeated use. The latter are much more easily prepared and are more convenient and economical in use. The bottles containing 10 ml. of carbol saline are autoclaved. The rubber stoppers (preferably the skirted type) are boiled in 5 per cent. phenol and are fitted as soon as the bottles are removed from the autoclave. The final dilutions, except in the case of alcohol killed vaccine, should always be made in carbol-saline. When we know the number of bacteria per ml. in the suspension, it is an easy matter to calculate how much carbol-saline should be removed from a bottle and replaced with the same amount of the suspension to give 10 ml. of vaccine of the required strength. Fluid is removed from or added to vaccine bottles with a sterile graduated syringe fitted with a fine needle which punctures the rubber cap after this has been treated with a disinfectant such as 5 per cent. phenol.

An autogenous vaccine is one prepared from the bacteria isolated from the patient's own lesion. A stock vaccine is prepared from the bacteria isolated from the lesion of another patient suffering from a similar condition or, more usually, from a number of strains of such bacteria. A mixed vaccine, that is one containing more than one variety of organism, is used in conditions such as chronic bronchitis or acne when it is believed that more than one organism may play a part in the ætiology of the condition.

Detoxicated vaccines are made from bacteria freed from their endotoxins by treatment with chemicals or by disintegration in a mill. The fact that enormous doses of these vaccines can be given without severe reactions appeals to some people. Besredka introduced sensitised vaccines which were prepared by treating vaccines with their antisera and then centrifuging and washing the treated bacteria. They consist of bacterial antigens combined with their antibodies, the purpose of which is to reduce the toxicity of the antigens. Besredka was also responsible for the introduction of the oral method of administration of vaccines. He claimed that if a bile pill were swallowed, the mucous membrane of the intestine was rendered capable of absorbing the bacteria in the vaccine and of developing a local immunity superior to the general immunity resulting from the subcutaneous administration of a similar vaccine. Few workers are prepared to accept Besredka's views.

Vaccines may be used: prophylactically or therapeutically. There can be no doubt of the efficacy of vaccines as prophylactics of certain diseases. From one to five subcutaneous injections, depending on the organism, at intervals of 1 to 2 weeks give, against a number of diseases and particularly the enteric fevers, an immunity which can be demonstrated in the laboratory by an increase in the amount of specific antibody in the subject's serum and, much more important, which protects against all but massive doses of the pathogenic organism. For the preparation of prophylactic vaccines, cultures of freshly isolated and fully virulent organisms should be used and care should be taken not to damage their antigenicity by the method of preparation of the vaccine.

Vaccines have now been used therapeutically for many years and there can be no doubt that the number of honest, critical and competent persons who believe in their efficacy is very much fewer than it was in, say, the year 1910. Many of the successes attributed to vaccine therapy were probably due to a coincidental rather than consequential recovery from the disease and more still to the faith of the patient in vaccines, in the ritual of inoculation or in the inoculator. Their use in acute conditions has been almost completely abandoned, as was to be expected on theoretical

we require either a collection conditioned to a different type phage and a collection of cultures, with the aid of which the phage can be conditioned to each type of bacillus.

If we have a Type II phage, conditioned to Type F *Salm. typhi*, which we wish to condition to Type C bacilli, we add undiluted F phage and the same diluted 1 : 10 and 1 : 100 to areas spread with Type C bacilli and incubate. We select an area showing a few well separated plaques and, with a sterile knife, cut out a piece of the agar including some of the culture of bacilli and only one plaque. We drop this into broth and incubate for 18 to 24 hours. Then, after heating to 58° for 30 minutes, which is sufficient to kill any surviving typhoid bacilli but not the phage, we make serial dilutions of this (1 : 10, 1 : 100 and so on up to 1 : 10¹² in broth), add to each an equal volume of a young culture of Type C bacilli, and incubate for 18 hours. The last clear tube in the series

for 30 minutes. Phages can be preserved for months in the refrigerator.

We next determine the Critical Test Concentration (C.T.C.) of the phage of the type of the strain. The reason that it is always necessary, in typing strains, to use the phage at its C.T.C.

Before we attempt to type a strain of *Salm. typhi*, we must find out if it is in V form, as Type II phages act only on V forms (i.e. those containing V₁ antigen). We discover this by testing it with some of the other phages (III and IV) which lyse V forms of

Salm. typhi, irrespective of type, but not W forms which do not contain Vi antigen or transitional forms which contain little of it.

Having satisfied ourselves that the new strain is of V form, we carry out the test. On an agar plate we spread a number of areas with a young broth culture of the organism in the way already described and allow these to dry. To each we add a drop of a preparation of phage conditioned to a particular type of *Salm. typhi*, each diluted to its C.T.C. which has already been ascer-

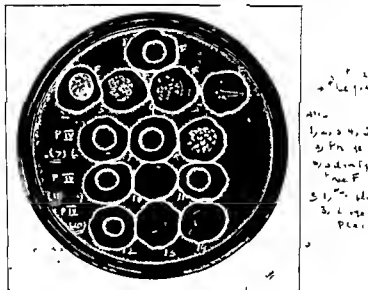


FIG. 28 — ACTION OF PHAGE ON *Salm. typhi*

tained. After incubation it will be seen that only one area shows confluent lysis, the others showing at most isolated plaques. The organism under test belongs to the same type as that to which the phage causing confluent lysis belongs.

Similar methods are used for the phage typing of staphylococci and other bacteria.

Fig. 28 illustrates the results obtained in carrying out some of the procedures described in this section.

To type a strain of typhoid bacillus we require either a collection of phages, all of Type II, each one conditioned to a different type of *Salm. typhi*, or a single Type II phage and a collection of cultures of various types of *Salm. typhi*, with the aid of which the phage can be conditioned to each type of bacillus.

If we have a Type II phage, conditioned to Type F *Salm. typhi*, which we wish to condition to Type C bacilli, we add undilute F phage and the same diluted 1 : 10 and 1 : 100 to areas spread with Type C bacilli and incubate. We select an area showing a few well separated plaques and, with a sterile knife, cut out a piece of the agar including some of the culture of bacilli and only one plaque. We drop this into broth and incubate for 18 to 24 hours. Then, after heating to 58° for 30 minutes, which is sufficient to kill any surviving typhoid bacilli but not the phage, we make serial dilutions of this (1 : 10, 1 : 100 and so on up to 1 : 10¹² in broth), add to each an equal volume of a young culture of Type C bacilli, and incubate for 18 hours. The last clear tube in the series

for 30 minutes. Phages can be preserved for months in the refrigerator.

We next determine the Critical Test Concentration (C.T.C.) of the phage of this type of *Salm. typhi*. The reason that it is always necessary, in typing strains, to use the phage at its C.T.C.

Before we attempt to type a strain of *Salm. typhi*, we must find out if it is in V form, as Type II phages act only on V forms (i.e. those containing V₁ antigen). We discover this by testing it with some of the other phages (III and IV) which lyse V forms of

By the use of phage IV it is shown that the three strains are all V₂ forms and should, therefore, be capable of being typed. In the case of strain 7, phage II F produced confluent lysis and phage II C lysis falling considerably short of being confluent. Strain 7 is of type F. These results show the importance of using phage preparations at their C.T.C. Unless a strain is completely lysed by a phage preparation at its C.T.C., it does not belong to that type. The fact that it is partially lysed by a preparation of phage of a different type at its C.T.C. is of no importance in typing.

Strain 11 is of phage type C.

Strain 20 does not belong to either type F or type C. In order to type it, other phage preparations must be used

Loopfuls of young broth cultures of various strains of *Salm. typhi* were deposited as described above at fourteen points on the surface of the agar in a large plate. These have produced irregularly circular areas of growth.

In the first five of these:

1
2 3 4 5

the organism used was *Salm. typhi* of phage type F. To each of these areas (after drying and before growth had occurred) was added a loopful of a dilution of a preparation of phage Type II F, the dilutions used being:

No. 1	.	.	1 : 10 ³ - C. T. C.
No. 2	.	.	1 : 10 ⁴
No. 3	.	.	1 : 10 ⁵
No. 4	.	.	1 : 10 ⁶
No. 5	.	.	1 : 10 ⁷

No. 1 shows confluent lysis, No. 2 almost confluent lysis, No. 3 a large number of plaques, No. 4 a smaller number of plaques and No. 5 two plaques. The C.T.C. of the preparation of phage II F for its homologous organism is, therefore, 1 : 10³.

The nine other areas show the typing of three strains of *Salm. typhi* (Nos. 7, 11 and 20). A preparation of phage IV and preparations of phage II conditioned to types F and C were used, each at its C.T.C. The results obtained were as follows:

Strain of (<i>Salm. typhi</i>)	Phage (C.T.C.),		
	IV	II F C.T.C.	II C
7 F	C. L. (6)	C. L. (7)	P (9)
11 c.	C. L. (1)	Nil (1)	C. L. (1)
20	C. L. (12)	Nil (1)	Nil (1)

C. L. = confluent lysis

P = plaques only

Nil = no action.

A. The Slide Method

This method is used to assist in identifying an organism. Suppose we have cultured the faeces of a suspected typhoid carrier and we find on a MacConkey plate a number of pale colonies resembling those of *Salm. typhi*. We take a clean microscope slide and, with a Dreyer pipette, put one drop of saline on it. A portion of one of the colonies is picked up with a platinum loop and rubbed up in the drop so as to make an even suspension. To this suspension one loopful of an anti-typhoid serum is added and the slide is rocked for a few seconds. If the organism is the



FIG. 29.—SLIDE AGGLUTINATION

A culture of *Salm. typhi* was mixed with drops of antisera prepared against *Salm. typhi* (T), *Salm. paratyphi* A (A), and *Salm. paratyphi* B (B). It has been agglutinated by T but not A or B.

in less
visible
If the
organism is not the typhoid bacillus, or a very closely related *Salmonella*, the suspension will remain uniform without any floccules forming. Individual colonies are tested in this way and a subculture is made for further investigation from one which is found to be agglutinated by the serum.

The method is not to be taken as more than a rough and rapid one, suggesting rather than establishing the identity of the organism under investigation, but within its limitations it is very useful.

CHAPTER VII

SEROLOGICAL TECHNIQUE

This chapter, which deals with the practical aspects of serology, should be read in conjunction with the theoretical consideration of the subject given in later chapters.

Agglutination Tests

The agglutination of bacteria by antisera may be used to assist in the diagnosis of disease either:

1. By mixing the serum of the patient with a suspension of known bacteria and so determining whether the former contains antibodies acting on the latter.
2. By mixing the serum of an animal which had been immunised with a known organism, with a suspension of the bacterium isolated from the patient and so determining whether the bacterium is identical with that used to immunise the animal.

There are four methods of carrying out agglutination tests:

- A. Slide method.
- B. Microscopic method.
- C. Dreyer's macroscopic method.
- D. Felix's macroscopic method.

In the following pages questions of dilution are frequently mentioned. When a dilution of serum in saline, for example, is stated to be 1 : 5, this means that one part of serum is present in every five parts of the diluted preparation. 1 part serum + 4 parts saline = 5 parts of 1 : 5 dilution.

The titre of an agglutinating serum is the highest dilution of the serum which causes agglutination of the bacteria against which it is tested. It may be expressed as a vulgar fraction (e.g. 1/100) or, better, as a proportion (e.g. 1 : 100).

A. The Slide Method

This method is used to assist in identifying an organism. Suppose we have cultured the faeces of a suspected typhoid carrier and we find on a MacConkey plate a number of pale colonies resembling those of *Salm typhi*. We take a clean microscope slide and, with a Dreyer pipette, put one drop of saline on it. A portion of one of the colonies is picked up with a platinum loop and rubbed up in the drop so as to make an even suspension. To this suspension one loopful of an anti-typhoid serum is added and the slide is rocked for a few seconds. If the organism is the



FIG. 29 —SLIDE AGGLUTINATION

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typhoid bacillus the suspension will become lumpy and in less visible. If the organism is not the typhoid bacillus, or a very closely related *Salmonella*, the suspension will remain uniform without any floccules forming. Individual colonies are tested in this way and a subculture is made for further investigation from one which is found to be agglutinated by the serum.

The method is not to be taken as more than a rough and rapid one, suggesting rather than establishing the identity of the organism under investigation, but within its limitations it is very useful.

To give it maximum reliability certain precautions must be taken.

way described above. Some experience is required to secure the optimum density of the suspension on the slide. It should be rather denser than a suspension made to prepare a film for staining. By using the serum in the dilution suggested, false positive results due to insufficiently diluted serum acting on organisms



FIG 30 — MICROSCOPIC AGGLUTINATION.

related to, but not identical with, the organism against which the serum was prepared will be, to some extent, avoided, as also will false negative results due to excessively diluted serum. It is a good practice always to make two identical suspensions on a slide and to add the serum only to one, the other being kept as a

anti-haemagglutinating (H) and the organism is non-motile or if the serum is anti-somatic (O) and the organism is either rich in Vi antigen or has lost its O antigen. Motile bacteria are richest in H antigen when grown in broth, but broth cultures are not sufficiently dense

for the slide method. ²When grown on agar, especially if its surface is dry, and when recently isolated on selective media, H antigen may be partially or completely deficient.

B. Microscopic Method

This method, which was that of the original Widal test, is now rarely used. ³Various dilutions of the serum under examination are mixed with ²equal volumes of a broth culture of the organism

lose their motility. The serum of the when diluted 1/50 or 1/100. Accurate estimations of the titre of a serum by this method are impossible.

C. Dreyer's Macroscopic Method

This method is most useful when the ⁴organism is motile and

broth culture of an actively motile strain, treated with 0.1 per cent formalin to kill the bacilli and diluted with saline so as to contain from 250 to 400 million bacilli per ml., forms a suitable suspension which keeps well in the cold. ⁵Two types of tubes are used—8.5 × 0.75 cm. round bottom tubes for diluting the serum and 6.0 × 0.5 cm. tubes with conical ends for carrying out the agglutination. Measurements are made in terms of drops, a special type of pipette—Dreyer's—being used. To obtain drops of uniform size the rate of dropping must be slow and the pipette held vertical.

A 1 : 10 dilution of the serum is made in one of the larger tubes. If to 18 drops of saline 2 drops of serum are added (or any other volume in a 9 : 1 ratio), the serum will be diluted 1 : 10. The small tubes are arranged in series of 5. To those in one series are added 0, 5, 8, 9 and 10 drops of saline followed by 10, 5, 2, 1 and 0

drops of 1 : 10 serum. The pipette is well washed and charged with the suspension of which 15 drops are added to each tube. Every tube now contains a total of 25 drops made up as follows:

Tube No.	1	2	3	4	5
Saline	0	5	8	9	10
1 : 10 Serum (4 to body)	10	5	2	1	0
Suspension (4 to tube)	15	15	15	15	15

So yielding:

Final dilution of serum 1 : 25 1 : 50 1 : 125 1 : 250 0

The four tubes give serum dilutions ranging from 1 : 25 in the first tube to 1 : 250 in the fourth. The fifth tube acts as a control to show that the suspension has not spontaneously agglutinated.

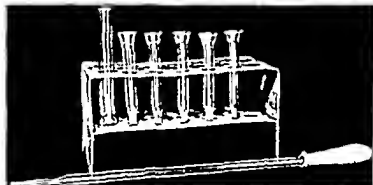


FIG. 31.—APPARATUS FOR AGGLUTINATION TEST (DREYER'S METHOD).

Where the titre of the serum is higher than 1 : 250, a second similar series is put up using a 1 : 200 dilution of the original serum (19 drops of saline + 1 drop of 1 : 10 serum or in this proportion). This yields dilutions ranging from 1 : 500 to 1 : 5000. A third series may similarly be set up using 1 : 4000 serum (19 drops of saline + 1 drop of 1 : 200 serum, or in this proportion) and giving dilutions of 1 : 10,000 to 1 : 100,000.

Where this method is used for the diagnosis of enteric fever in unimmunised patients, to carry dilutions further than

enteric fever may be due either to *Salm. typhi*, or to one of the other salmonellæ, it may be necessary to test the serum against several of these. In the British Islands, enteric fever is almost invariably due to either *Salm. typhi* or *Salm. paratyphi B* and so only exceptionally need other organisms be considered. Two series, identical as regards the amounts of saline and 1 : 10 serum but varying in the suspension are used. To each tube of the first series are added 15 drops of the typhoid suspension and to each

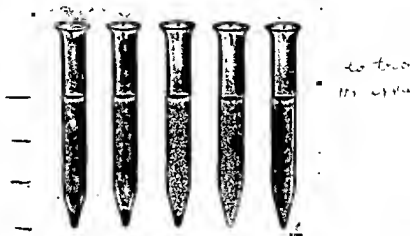


FIG 32 —MACROSCOPIC AGGLUTINATION TEST (DREYER'S METHOD)

Complete agglutination is present in the first two tubes, intermediate in the third and standard in the fourth. The fifth (control) tube shows no agglutination.

of the second series 15 drops of the paratyphoid B suspension. The contents of each tube are mixed and the rack is placed in a water bath at 52° , the level of the water being such that only about one third of the column of fluid in the tubes is submerged. The object of this is to secure the maximum of mixing caused by convection currents.

After 2 hours in the water bath, the rack is removed and the tubes are examined individually within 10 minutes. Observation is facilitated if the tubes are held against a black background and

illuminated from above and behind. A special viewing box supplies suitable illumination. Tubes should be examined without magnification. Early tubes in a series may show complete agglutination, all the bacilli being sedimented in a flocculent mass at the bottom of the tube leaving the supernatant fluid quite clear. Later tubes may show marked agglutination with some sedi-

... d at different times
 s ✓ same serum might
 h suspension and of
 1 : 500 when tested with another. This may be allowed for by calculating a factor for each suspension which, when multiplied by the actual titre, gives the standardised titre. A simpler method is to reject suspensions varying widely from the one accepted as standard.

D. Felix's Macroscopic Method

This method is used to determine the O (anti-somatic) or Vi titre of a serum.

A series of 8 round bottom tubes (7.5 × 1.1 cm.) is taken and 0.8 ml. of saline is measured into the first and 0.5 ml. into each of the others. With a fresh pipette, 0.2 ml. of serum is measured into the first tube and mixed thoroughly with the saline already in the tube by drawing into the pipette and expelling from it several times. With the same pipette, 0.5 ml. is transferred from the first to the second tube the contents of which are mixed in the same way. This process is carried on to the seventh tube from which 0.5 ml. is rejected. To each tube is added either 0.5 ml. of a 250-400 million per ml. suspension of the organism or 0.5 ml. of saline and 1 drop from a Dreyer pipette of a concentrated suspension (2500-4000 million per ml.). Thus we have a series of tubes in which the serum is diluted 1 : 10 in the first, 1 : 20 in the second, 1 : 40 in the third and so on, the eighth being a control

without serum. Since we are interested in the O or Vi titre of the serum, a living suspension of a non-motile strain may be used or, as is preferable, a suspension of an organism killed either with alcohol, to destroy flagellar but not somatic antigens, or with mercuric iodide to preserve the Vi antigen.

The contents of the tubes are mixed by shaking the rack containing them and this is then incubated at 37° for 2 hours. The rack is then left overnight at air temperature or, in warm weather in a cold room or refrigerator to prevent growth of bacteria, where it will not be disturbed and where there will be no irregularities of air temperature to cause convection currents in the tubes. During this period the bacteria deposit to the bottom of the



FIG. 33 —DEPOSITED BACTERIA (FELTZ'S METHOD)

1 Complete agglutination 2 Partial agglutination 3 No agglutination.

tube and what we observe is the pattern they form there. The tubes are held up above eye level somewhat tilted, their open ends being directed to the brightest part of the sky and their rounded bottoms are examined with a hand lens. Another method of examining the pattern formed by the deposited bacteria is to observe its magnified image in a concave mirror, such as that of a microscope. Unagglutinated bacteria are deposited, in a small dense disc with a sharp margin, at the centre of the bottom of the tube. Those completely agglutinated are aggregated in masses easily visible with the lens. These masses are deposited over the whole of the round bottom of the tube where they form an almost uniform carpet. In some cases the carpet has extended almost to the vertical side of the tube from which it has slipped down

towards the lower part, presenting a wrinkled margin. Lesser degrees of agglutination present a circular disc surrounded by granules of agglutinated bacteria.

Absorption of Agglutinin

Simple agglutination carried to the end-point of the serum is usually sufficient to identify an organism. Where, however, there is an appreciable antigenic overlap between two related organisms, antiserum to one may agglutinate the other to the same or even to a higher titre so that simple agglutination fails to distinguish between them. In such a case an absorption technique must be used. In a d serum, the antigenic

will be removed, its homologous organism while having no effect on the heterologous type. An example demonstrating the difference between the flagellar antigens of *Salm. enteritidis*, which are "g" and "m" and those of *Salm. dublin* which are "g" and "p", will make this clear. *Salm. enteritidis* antiserum contains agglutinins against antigens "g" and "m" and, because "g" is shared by *Salm. dublin*, agglutinates both strains to approximately the same titre. Absorption of this serum with homologous suspension removes both these agglutinins, so that the serum does not agglutinate either organism. Absorption with *Salm. dublin*, however, removes agglutinin against "g" but not against "m", since this antigen is

each, simple absorption of serum against either type with heterologous suspension is sufficient to establish the distinction between them. This is not the case when only one of the organisms possesses an additional specific antigen. For example, the flagellar

phase II of *Salm. paratyphi B* contains antigens 1, 2 and that of *Salm. typhimurium* antigens 1, 2, 3. The somatic antigens of these

against antigens 1 and 2, with either homologous or heterologous suspension, removes all agglutinin from it so that the two organisms still appear identical. If, however, *Salm. typhimurium* serum is absorbed with *Salm. paratyphi B* suspension, agglutinin against antigen 3 is not removed and so the treated serum agglutinates only *Salm. typhimurium*. It has, therefore, been demonstrated that *Salm. typhimurium* possesses all the antigens of *Salm. paratyphi B* together with an additional one of its own. Such an experiment, where antisera prepared against each of two organ-

serum to give an homologous titre of $\bar{Y} : 250$ prior to absorption. To about 50 ml. of a very dense suspension of the organism, obtained by washing off the growth from half a dozen agar slopes in a few ml. of saline, is added 0.5 ml. of the diluted serum. The mixture is allowed to stand in the incubator or water bath at 37° , for from 1 to 2 hours when absorption should be complete. It is then centrifuged to deposit all bacteria, the supernatant, absorbed serum is pipetted off and its volume made up to 10 ml. with saline. This gives a $\bar{Y} : 20$ dilution of absorbed serum which is then tested by Dreyer's or Felix's method against both the homologous and the heterologous organism. If the latter, which was used for absorption, is agglutinated, absorption has not been adequate and must be repeated using a denser suspension.

Grouping of Hæmolytic Streptococci

It is difficult to carry out serological identification by agglutination methods with bacteria, such as streptococci, which undergo spontaneous clumping. Serological identification is best effected in such cases by precipitation rather than agglutination and this is the method which should be used in determining to which of Lancefield's groups a hæmolytic streptococcus belongs.

It is necessary to have a supply of antisera each of which is the serum of a rabbit immunised against streptococci known to belong to a particular group. It is usually sufficient to have antisera for groups A, B, C, D and G which are those most commonly found in relationship to the human body.

The streptococcus to be grouped is grown in glucose broth for 48 hours. At the end of this time most of the cocci will have deposited in the bottom of the tube. The supernatant broth is poured carefully off into disinfectant and the deposit is transferred to a centrifuge tube in which it is centrifuged. The supernatant is removed as completely as possible with a fine pipette. The deposited streptococci are treated as follows:

1. Add 0.1 ml. Formamide.
2. Heat in an oil bath at 150° for 20 minutes.
3. Cool and add 0.25 ml. acidified alcohol. (Acidified alcohol is prepared by adding 5 parts of 2 N. HCl. to 95 parts absolute alcohol.)
4. Centrifuge and transfer supernatant fluid to another tube.
5. To the supernatant fluid add 0.5 ml. acetone.
6. Centrifuge. Carefully remove the supernatant fluid with a fine pipette and discard.
7. To the deposit add 1.0 ml. saline.
8. Centrifuge and transfer the supernatant fluid to another tube with a fine pipette.
9. To the supernatant fluid add a drop of phenol red solution and weak Na_2CO_3 solution until the colour is orange, indicating neutrality.

The final fluid obtained by this treatment is a relatively pure solution of the capsular polysaccharide which is the haptan

responsible for the group characteristics of the organism. If it is mixed with an antiserum prepared by immunising an animal with

their combining proportions. A gross excess of one either prevents the formation of a precipitate or leads to its solution almost as soon as it is formed. For this reason, this precipitation test, as well as many others, is best performed as a ring test, the solution of hapten being layered on the surface of the serum. Either above or below the interface the two reagents will, as a result of diffusion, find a level at which optimum proportions exist and at that point precipitate forms.

The best way to carry out the test is in a small tube 50×5 mm. Four drops of the extract are placed in the tube and four drops of serum are added. The latter addition is made with the tube slanted so that it is at an angle of rather less than 30° with the horizontal. The drops of serum are allowed to fall on the inner surface of the tube a short distance above the level of the extract. The serum, being denser than the extract, flows down the side of the tube, through the column of extract and comes to rest at the bottom of the tube. When the tube is held upright it will be found that there is a sharp line of demarcation between the serum below and the extract above. Similar preparations are made with the extract and each of the sera available.

The tubes are kept at air temperature for half an hour, when the results are read. In the one in which the hapten and antibody correspond, a marked precipitate will be seen at or close to the interface between the two fluids. No such precipitate should be present in any other tube. The streptococcus is of the same group as the one against which the serum giving a precipitate was prepared.

Hæmolytic Tests

The principal facts concerning the mode of action of hæmolytic sera can be very simply demonstrated.

(1) Collect some blood from a sheep into citrate solution to prevent clotting. Centrifuge, discard the plasma and wash the corpuscles. Re-suspend the cells in saline, making about a 2 per cent. suspension.

(2) Collect some blood from a rabbit which has received, on two or three occasions, intravenous injections of a suspension of washed sheep cells. Allow to clot and separate the serum.

(3) Collect blood from a normal guinea-pig, allow to clot and separate the serum.

Four small test tubes 9 cm. \times 1 cm. are taken and the following reagents are added, measurements being made in millilitres.

	<u>1</u> 1.0	<u>2</u> 1.0	<u>3</u> 1.0	<u>4</u> 0.5
Saline	1.0	1.0	1.0	0.5
Fresh serum of the immunised rabbit (diluted 1:20) }	0.5	—	—	—
The same diluted serum heated to 56° for 10 minutes }	—	0.5	—	0.5
Fresh serum of normal guinea-pig (diluted 1:10) }	—	—	0.5	0.5
Suspension of sheep cells }	0.5	0.5	0.5	0.5

The tubes are shaken and incubated in a water bath at 37° for half an hour. The first and fourth will have transparent red fluids, showing that hæmolysis has occurred. The second and third will still be turbid and, if left for some time, the uninjured red cells will sink to the bottom of the tube, leaving the fluid above

either blood cells or complement are employed: higher temperatures damage both. Note also that isotonic saline (0.85 per cent.

sodium-chloride) is always used in the presence of red blood cells as these would be hæmolyzed by water.

Wassermann Reaction

It may be well first to outline the principles on which this rather

complement). If the patient is syphilitic, the complement will be used up or fixed: if not, it will remain free as there is, in the mixture, no antibody to combine with the antigen. After a time we add red blood cells and the heated serum of an animal immunised against these cells and, therefore, containing hæmo-

test is negative, and the patient is not syphilitic. If, on the other hand, the cells are not hæmolyzed, the test is positive and the patient is syphilitic.

There are many methods of performing this test, but the one here given (which is based on Harrison's) is, in our opinion, one of the most reliable. The reagents required are: —

- (1) Suspension of washed sheep's red blood corpuscles—see above.
- (2) Hæmolytic serum (immune body, I.B.)—see above.
- (3) Fresh guinea-pig serum (complement)—see above.
- (4) Antigen.—This is prepared as follows. Take a fresh, healthy human heart; free the muscle from fat, and grind 10 g. with dry clean sand and 9 ml. of absolute alcohol. Let the mixture stand for 1½ hours, shaking occasionally; then filter through

filter paper. This constitutes the heart extract. A solution of

serum is heated to

In addition, when a re-test known positive and negative sera as controls.

So many reagents are employed in the test that it is necessary to standardise each as thoroughly as possible. The first to be considered is the blood suspension. Many workers are content to dilute the sedimented cells to a definite extent (e.g. to make a 5 per cent suspension); but we prefer to standardise by the content of hæmoglobin, which may be compared with the standard tube of a Haldane's hæmoglobinometer. In our practice the suspension is of such a strength that when it is diluted $\frac{1}{2}$ with distilled water and coal-gas passed through, the resulting solution of carboxyhæmoglobin exactly matches the Haldane's tube.

Titration of Immune Body

The immune rabbit's serum (I.B.), which has been heated to 56° for half an hour, is diluted, say, 1 : 1000, and to a series of tubes are added the following reagents measured in millilitres:

	1.	2.	3.	4.	5	(6)	7.
Saline	0.0	0.25	0.5	0.6	0.7	0.8	0.85
I.B. (1 : 1000)	1.0	0.75	0.5	0.4	0.3	0.2	0.15
Fresh guinea-pig serum (diluted 1 : 10)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Standard blood suspension (diluted 1 : 2 with saline)	0.5	0.5	0.5	0.5	0.5	0.5	0.5

The contents of the tubes are mixed and the rack incubated in the 37° water bath for half an hour. If the hæmolytic serum is a good one it will probably be found that tubes 1 to 6 show complete hæmolysis (i.e. the fluid is quite clear without any turbidity),

The contents are mixed and the tubes incubated at 37° for half an hour. The earlier tubes will probably show complete hæmolysis,

1 M.H.D. of complement is present in	0.28 ml. of 1:40 guinea-pig serum	
	= 0.007 „ undiluted „ „	
∴ 3 „ „ „	0.021 „ „ „	

so, in order to prepare a dilution of guinea-pig serum such that each 0.5 will contain 3 M.H.D. of complement, we must dilute 0.021 ml. up to 0.5 ml. with saline or, to make a convenient amount, we must dilute 0.84 ml. of the serum with saline to make 20.0 ml. Again—

1 M.H.D. of complement is present in	0.28 ml. 1:40 guinea-pig serum	
	= 0.007 „ undiluted „ „	
∴ 5 „ „ „	0.035 „ „ „	

To prepare the 5 M.H.D. dilution of complement we dilute 0.035 ml. of guinea-pig serum up to 0.5 ml. with saline, or 0.7 ml. up to 10.0 ml.

Since the serum of a fresh guinea-pig is used on each occasion when tests are performed, it is always necessary to titrate the complement as a preliminary to the test proper and to prepare the 3 and 5 M.H.D. dilutions.

The Test

For testing each serum three tubes are employed placed one behind the other. Into the three tubes the following reagents are measured, a fresh pipette being used for measuring each serum. It is usual to measure out the serum to be tested into the tubes first, then to add the necessary saline to all, and subsequently the complement and antigen.

	Front.	Middle.	Back
(1) Patient's serum (heated to 56°)	0 1 cc	0 1 cc	0.1 cc
(2) Saline	0 4	0 4	0 9
(3) Complement 3 M.H.D.	0.5	—	0 5
(4) Complement 5 M.H.D.	—	0 5	—
(5) Antigen	0 5	0 5	—

The tubes are shaken, left at room temperature for half an hour and incubated in the water bath at 37° for the same time. Then

serum procured for testing. The front and middle tubes differ only in that the front contains 3 doses of complement and the middle 5. If the reaction of the serum be positive, the complement will have been fixed in the first stage of the test and the tubes will show no hæmolysis. If negative, the complement will have been left free and hæmolysis will be complete. In the case of a reaction weaker than full positive it may be found that the middle tube shows complete or partial hæmolysis, and the front, with only 3 M.H.D. of complement, no hæmolysis. Owing to the presence of the two tubes containing different strengths of complement we can determine, to some extent, the strength of the reaction. Various methods of recording the results are used. In the first, two symbols are employed, the first of which shows the result obtained in the tube containing 3 M.H.D. of complement and the second in that containing 5 M.H.D. + signifies a positive reaction (no hæmolysis), ± or ± a partial positive (partial hæmolysis), and — a negative reaction (complete hæmolysis). The second system is usually preferred by the clinician, while the third is that recommended by the League of Nations Health Committee.

First system.	Second system.	Third system.	Interpretation.
++	+ 4 }	++	Full positive
+ ±			Strong positive
+ —	+ 3	+	Positive
± —	+ 2 }	±	Weak positive
± —	+ 1 }		Doubtful
— —	0	—	Negative

The test which now bears his name was elaborated by Wassermann on the theoretical basis that there should be present, in the blood serum of a syphilitic patient, antibodies to the causative organism and that, if such serum was mixed with a suspension of *Tr. pallidum*, the union of antigen in the spirochæte and antibody in the serum should fix complement. At that period the *Tr. pallidum* had not been cultivated, and so Wassermann took as his source of spirochætes an emulsion of the liver of a syphilitic fœtus which is very rich in these organisms. The test very soon demonstrated its value as a diagnostic agent and even the discovery that an emulsion, or still better an alcoholic extract, of normal organs, human or animal, could be substituted for the material containing spirochætes did not discredit it. Since the theory on which Wassermann based the test has been shown to be wrong, the test might be regarded as merely empirical, but vast experience of its reliability forces us to attempt to explain how the test does work. It behaves as if there was a specific relationship between substances in the alcoholic extract of normal organs and what we must presume are antibodies in the patient's serum. Two theories have been advanced. One is that the lipoidal material extracted from the organs is identical with the lipoidal material present in the body of the spirochæte, the other is that, during syphilitic infection, there is an abnormal liberation from the tissues of the patient of the same lipoidal substance as is present in Wassermann antigen and the patient produces antibodies against this substance. Whether either of these is correct or not is largely a matter of theoretical interest. The important thing is that few diagnostic tests have as high a degree of reliability as the Wassermann reaction.

There are very many modifications of the Wassermann reaction. The aim in most of these is to render the reaction more sensitive, so that the serum of a syphilitic patient will not give a negative result. It is an easy matter to increase the sensitivity of the test, but increased sensitivity carries with it the disadvantage of reduced specificity.

Complement fixation tests for other diseases such as gonorrhœa and tuberculosis may be carried out in a similar fashion, the antigen usually being a suspension of the bacteria causative of the disease. In the case of many virus infections, a complement fixation test has been found of value

Kahn Test :-

A number of tests which have been introduced for the diagnosis of syphilis are based on the fact that, when the serum of a syphilitic is incubated with a Wassermann "antigen", flocculation appears in the mixture. Of these the Sachs-Georgi was the original, and among others are those of Meinicke and Vernes, and the Sigma Reaction of Dreyer and Ward.

Within the past 20 years, however, the Kahn test has established itself as the most reliable and most widely used of the flocculation reactions. For it only three reagents are required:

1. Patient's serum.—This is obtained in the same way as for the Wassermann reaction, and is heated at 56° for half an hour.
2. Saline.—0.85 per cent. sodium chloride solution
3. Antigen—This consists of an alcoholic extract of beef heart (previously treated with ether) to which 0.6 per cent. of cholesterol is added. The details of the preparation cannot be described here, and those interested are referred to "The Kahn Test" by R. L. Kahn. The prepared antigen can be purchased through the usual channels. It must be kept in bottles with tightly fitting stoppers or rubber bungs covered with tinfoil. For use the antigen is diluted with saline, the degree of dilution needed for any particular batch of antigen being described as its titre. Usually 1.0 ml. of antigen requires from 1.0 to 1.3 ml. of saline.

To carry out the test 1.0 ml. of antigen is measured into a 5.5

cm. \times 1.5 cm. flat-bottom vial, and the amount of saline indicated by the antigen's titre (say 1.2 ml.) into another similar vial. The saline is poured into the vial containing antigen, and the mixture rapidly poured back. This double operation is repeated six times rapidly, in no case waiting to drain a vial completely. The antigen so diluted is allowed to stand for 10 minutes before using: it must not be used more than 30 minutes after dilution. It is then well shaken, with the thumb on the end of the vial, and measured into the tubes used for the test. These tubes measure 7.5 cm. \times 1.0 cm. Three tubes, arranged one behind the other in a rack, are used for each serum tested. 0.05 ml. of dilute antigen is measured into the front tube, 0.025 ml. into the middle, and 0.0125 ml. into the back, finely graduated pipettes being used and the amounts being delivered to the bottom of the tubes. Without delay (*i.e.* as soon as the antigen has been measured into the ten sets of three tubes which the rack holds) 0.15 ml. of each serum is measured into each of the three tubes used for testing that serum, and the rack is vigorously shaken by hand for 10 seconds. Where the air temperature of the laboratory is below 21° (as it usually is in Europe in the winter), it is now advisable to place the racks in a water bath at 37° for 10 minutes. With higher air temperature this is unnecessary. The racks are then vigorously shaken (by hand or in a machine) for 3 minutes at from 275 to 285 oscillations per minute. 1.0 ml. of saline is added to each front tube, and 0.5 ml. to each middle and back tube, and the racks shaken by hand sufficiently to mix the contents of the tubes. The results may then be read. In strongly positive reactions, flocculation is easily visible to the naked eye, even without removing the tube from its rack. In negative reactions the fluid is uniformly opalescent. For reading results we prefer holding the tube almost horizontally about $1\frac{1}{2}$ inches above a concave mirror and looking at the image in the mirror. This gives good illumination and sufficient magnification. For each serum tested the reactions occurring in the three tubes are recorded on the following scale:

++++ = particles easily visible to naked eye.

+++ = smaller particles but still visible to naked eye.

++ = fine particles on the border-line of visibility without magnification.

+ = particles visible only with magnification.

± = doubtful.

— = uniform opalescence.

The strongest reactions may occur in either the front or back tube of the three. To obtain the final result for the serum the number of + signs recorded for the three tubes is added and divided by 3. In case this gives a fraction, if the fraction is $\frac{1}{3}$, it is disregarded, if $\frac{2}{3}$ an extra + is added to the whole number.

Front.	Middle	Back		Final Result
+++	+++	+++	=	+++
+	++	++	=	++
+++	+	—	=	+
+	+	++	=	+
—	—	+	=	—

++ or over is regarded as positive, + or ± as doubtful, and — as negative.

It is important to note that the reaction is not a flocculation reaction.

The reaction is not a flocculation reaction.

The reaction is not a flocculation reaction.

The reaction is not a flocculation reaction.

tested an amount of antigen approximating to the optimum.

It occasionally, but fortunately only rarely, happens that the amount of antibody in the serum is so excessive that it is grossly disproportional even to the largest amount of antigen. In such cases the reaction may be weak or even negative. If serum, diluted 1 : 5 with saline, is used, good flocculation will occur.

The Kahn test has at least as high a degree of reliability as the best of the modifications of the Wassermann reaction and, since the number of reagents required is much smaller, mechanical errors are less likely to occur with it. It is highly advisable to carry out both tests on every serum submitted for the diagnosis of syphilis and to repeat both tests when the results are discrepant.

If the Wassermann reaction is positive and the Kahn negative, the Kahn test should be repeated using serum diluted as described above. A serum is occasionally found to give, on repeated tests, a negative Wassermann and a positive Kahn test. In such cases the Kahn verification test should be invoked. This is carried out in the same way as the ordinary Kahn test except that it is set up in duplicate. One set of reagents, tubes and pipettes is chilled to 1° , and the whole test is performed at a temperature as close to that as possible. The other set is heated to 37° , at which temperature the test is conducted. If the patient from whom the serum was obtained is syphilitic, the test at 37° will be positive and that at 1° either negative or much less strongly positive than when the test was performed at air temperature. Non-specific reactions are usually stronger at 1° than at 37° .

In early diagnosis and in assessing the effects of treatment, it should be remembered that the Wassermann test usually becomes positive earlier than the Kahn, while the positive Kahn persists longer following treatment.

CHAPTER VIII

THE OBTAINING OF MATERIAL FROM THE PATIENT FOR BACTERIOLOGICAL EXAMINATION

The ultimate aim of much of our practical bacteriological work is to be able to identify the organism responsible for some pathological condition. The ubiquity of bacteria must be borne in mind in collecting material for bacteriological examination. We wish to obtain the pathogen responsible for the disease; we must be on our guard to exclude, so far as possible, bacteria which are saprophytic or merely parasitic. Obviously, if we examine the faeces for pathogenic organisms, we cannot obtain a specimen without a large number of normal intestinal bacteria; but if we collect pus from an abscess we should avoid the bacteria occurring on the skin, or if we propose to examine urine we should endeavour to obtain that fluid as it exists in the bladder, avoiding the organisms found in the terminal portion of the urethra.

For the collection of material for bacteriological examination we should provide ourselves with a supply of sterile apparatus of which the most useful are plugged test tubes, cotton swabs on wires, capillary pipettes, and screw cap bottles. The methods of obtaining material from certain localities will now be mentioned.

Throat Swab

The patient's head is thrown slightly backwards, the tongue depressed with a spatula, and the patient asked to say "ah—ah—ah—ah!" In this way the soft palate is elevated, and the swab held by the wire is withdrawn from its tube and introduced into the mouth. It is rubbed firmly on the suspected part, whether the tonsil, supratonsillar fossa, uvula or elsewhere, withdrawn, avoiding the tongue and lips, and restored to its tube. If *C. diphtheria* is to be searched for, the swab should be used to inoculate a tube

of coagulated serum and also a plate or tube of a tellurite medium, both of which are then incubated. Gentian violet blood agar is to be recommended when streptococci are being searched for. If we merely desire information as to the general bacteriology of the part, a blood-agar plate is inoculated from the swab and subsequently spread. In all cases some direct films should also be made from the swab, and one of these may be stained by Gram's method. If this precaution is not taken, the occurrence of the organisms associated with Vincent's angina may be overlooked. Swabs from the naso-pharynx may be similarly obtained, but the swab should have its terminal centimetre bent almost at a right angle; this is hooked under the soft palate, which should not be touched, and

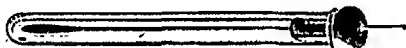


FIG. 34.—STERILE SWAB.

rubbed over the posterior naso-pharyngeal wall. A West's swab is sometimes used: this is made of thin, flexible wire and is protected by a glass tube the terminal portion of which is bent almost at a right angle. The swab is held within the tube until the soft palate is passed, when it is pushed out and rubbed over the wall of the naso-pharynx. It is then retracted within the protecting tube and the whole withdrawn. The obtaining of a swab from the larynx in cases of suspected laryngeal diphtheria should be left to a laryngologist.

Pus from an Abscess and other Fluids

The skin should be disinfected as thoroughly as possible and the disinfectant removed with alcohol. When the surgeon has made the opening the pus may be allowed to flow into a sterile tube if large in amount or, if not much is present, some may be

collected on a swab. Fluid such as that found in a vesicle may be collected in a sterile capillary pipette. The end of the pipette is then sealed in order to allow the fluid to be carried to the laboratory. In all cases where a swab is used, unless it can be examined with very little delay, it should, before the pus is collected, be moistened with broth to prevent drying. For an internal abscess a swab with a long wire handle is most useful for obtaining a sample of the pus. However obtained, films of the pus should be stained and cultures made on blood-agar or other medium suggested by the microscopic examination.

Sputum

The mouth should be rinsed out with warm boiled water (not containing any antiseptic) and the throat gargled with the same. By deep coughing, material should be brought up from the deeper parts of the respiratory tract and expectorated into a sterile wide-mouthed bottle provided with a screw cap or rubber bung. For examination, the sputum must be emptied into a sterile Petri dish and, if cultures are required, a selected mass of purulent material should be taken with the platinum loop and rinsed in three or four changes of sterile saline to free it from contaminating organisms on its surface. It may then be plated on blood-agar and preferably also on boiled blood-agar. A similar mass of purulent material should be used for making films for the detection of tubercle bacilli or other bacteria. Where little sputum is produced, a satisfactory specimen for microscopic or cultural examination may sometimes be obtained from the larynx by a laryngeal swab. Some persons, especially children, swallow most of the sputum they produce. In these, as indeed in all, examination for tubercle bacilli of material obtained by gastric lavage often yields a positive result. Such material is unsuitable for microscopic examination: culture or guinea-pig inoculation should be employed.

Fluid from Serous Cavities (Pleura, peritoneum, etc.)

This should be obtained with a syringe and needle, preferably an all-glass syringe autoclaved or dry sterilised ready assembled.

After the skin has been treated with iodine, the needle may be introduced into the cavity and the fluid aspirated. When the needle is withdrawn, the fluid should be expelled immediately into a sterile tube or screw cap bottle. When such fluid is almost clear (not purulent) it frequently clots, and this may interfere with its examination. It is therefore advisable to divide it into two portions, one of which is received in a dry sterile tube or bottle and the other in a sterile tube or bottle containing 1 to 2 ml. of citrate solution. If the fluid is clear or only slightly turbid, some of it should be centrifuged at a high rate for a few minutes and films made from the deposit. Information of value can frequently be obtained from observation of the types of leucocyte present. When, for example, lymphocytes predominate, it is unlikely that the infecting organism is of pyogenic type. Films should be stained by Gram's method for the detection of bacteria but, since the morphology of the leucocytes is not well seen in films stained by this method, additional films should be stained with methylene blue. Where Gram's method fails to reveal any bacteria, cultures should be made on blood-agar and a number of films examined for the presence of tubercle bacilli. Cerebro-spinal fluid is usually obtained with a special long needle 1 mm. in bore. This is introduced into the canal through the third or fourth lumbar space, the patient's back being bent so as to increase the distance between the vertebrae. The fluid emerging is collected in a sterile test tube. Since the first few drops frequently contain red blood corpuscles, it is well to collect the fluid in a series of two or three tubes, the latter of which will probably be free of these. The method of examining this fluid is similar to that described above, but examinations should be made quickly if the meningococcus is suspected. In the case of tuberculous meningitis, the tubercle bacilli can almost invariably be found by prolonged search of films made from the centrifuged deposit or from the fine fibrin web which forms on standing. Where this fails, and in the case of pleural fluid where failure is common, a guinea-pig should be inoculated with the deposit and a culture made on media suitable for the growth of the tubercle bacillus. In this way tuberculosis may

often be detected where microscopic examination has given negative results. The nature of the cells present is suggestive of the infective organism, since a predominance of lymphocytes in a clear fluid is usual in tuberculosis and of polymorphonuclear leucocytes in a turbid fluid in more acute infections. In very acute cases of tuberculous meningitis, however, polymorphonuclear leucocytes may predominate.

Urine

It is always preferable to obtain specimens of urine by a sterile catheter but, in the male, washing of the glans and meatus with soap and water, the rejecting of the first portion voided, and the collection of the latter part in a sterile flask, is quite satisfactory if the specimen is to be examined immediately. Catheterisation is essential in the female. Direct examination of a drop of urine from a case of cystitis will usually reveal pus cells and bacteria and a culture made from a loopful will generally give many colonies. In some cases, however, it may be necessary to centrifuge in order to obtain pus cells and bacteria in sufficient numbers for microscopic examination. Films made from urine should, after fixing, be well washed in water (which may be gently warmed on the slide by applying a small flame beneath). This removes much of the unorganised deposits. The films may then be stained as described. By doing this, clearer and more satisfactory microscopic preparations are obtained than by staining the film directly after fixing. Where the culture from the uncentrifuged urine shows only one or two colonies these are to be regarded with doubt. Plates of blood-agar and of MacConkey's medium permit the growth of most bacteria, except the tubercle bacillus, likely to be found in urine. The centrifuged deposit should be used to prepare films for the detection of tubercle bacilli. In tuberculosis of the urinary tract, these are generally fairly numerous; but one must be guarded since the smegma bacillus is commonly found about the external genitals and this organism is also acid-fast. The best precautions are thorough preparation of the part, the use of a catheter and, in the Ziehl-Neelsen method,

a full treatment with alcohol. In case of doubt, the only absolutely reliable method is the injection of a guinea-pig; this method has the further advantage over the direct microscopic examination that it gives a higher percentage of successes.

Fæces

The stool should be passed into a clean vessel and a small portion removed with the spoon supplied with the collecting outfit. If dysentery bacilli are sought, a portion of blood-stained mucus should be isolated, washed in several changes of sterile saline, and plated on desoxycholate-citrate medium, using dried plates. For members of the enteric and food-poisoning groups, a tube of tetrathionate broth should be inoculated heavily. Plates of Wilson and Blair's medium and of desoxycholate-citrate medium should also be spread. The tube should be incubated for 24 hours when plates of the same media should be spread from it. Wilson and Blair plates should be incubated for 48 hours, desoxycholate-citrate plates for 24 hours. The rapid growth of *V. cholerae* on the surface of alkaline peptone water is used to secure enrichment of that organism as a preliminary to plating. Streptococci may usually be isolated from fæces by culturing a small portion in glucose broth. The deposit after 24 hours' incubation should be plated on blood-agar. Tubercle bacilli are not commonly found in films made directly from fæces. Concentration methods give better results, but the only reliable method is to treat with an equal volume of 5 per cent. caustic potash or 3·4 per cent. sulphuric acid in order to kill other bacteria and to inject the deposit obtained by centrifuging into a guinea-pig.

In many cases, particularly in the examination of suspected carriers of the enteric bacilli, the previous administration of a purgative followed by salts will give a stool more likely to contain the organism than one obtained without purgation. The result obtained by a simple soap enema is also frequently useful for culturing the enteric bacilli from a patient or carrier.

Genitals

The commonest examinations made are for the organisms causing venereal disease, particularly the gonococcus and *Tr. pallidum*. The gonococcus, in the male, is present in the urethral discharge. The glans and meatus are well washed with sterile water or saline, and some pus is expressed by massaging the urethra from behind forwards, this is collected on a platinum loop and, if a culture is desired, is immediately spread on a plate of blood agar or boiled blood agar which is at once incubated in an atmosphere containing 10 per cent. of carbon dioxide. Films are made and stained by Gram's method. If no pus is obtained in this way, it may be provided by massaging the prostate from the rectum. In the female, material should be taken on a swab or platinum loop from the urethra, the cervix, or the mouth of Bartholin's gland, not from the vagina.

If a suspected chancre is present, it should be well cleansed with saline, squeezed vigorously and rubbed with sterile gauze. Some oozing of blood may occur, and this should be absorbed. Soon the blood ceases to flow and a drop of clear fluid appears. This is collected with a fine capillary pipette and used for examination by dark ground illumination or for preparing films for staining. Rubber gloves should be worn to prevent accidental infection.

Blood Culture

The syringe, an all-glass one with a really sharp needle, should be assembled, placed in a large glass test tube and sterilised in the autoclave or dry oven. Only in emergencies should boiling be relied upon. 100 ml. of broth (either ordinary broth or, if the enteric group be suspected, broth containing 0.5 per cent. sodium taurocholate, which inhibits the bactericidal action of the patient's blood) should be ready in a blood culture bottle which is a 6-oz. bottle with a perforated screw cap fitted with a rubber washer. A linen thread or fine wire is tied around the neck of the bottle and brought over the top, and a cellulose cap is placed over the metal cap. The whole is autoclaved, and it will be found, after drying, that the cap has contracted, forming a firm covering over the

metal cap and neck of the bottle, which prevents bacterial contamination of the exposed portion of the rubber washer. It is of importance to have as large a volume of broth as possible in the bottle so that any antibacterial substances present in the patient's blood, which might interfere with the growth of the bacteria outside the body, may be diluted to such an extent as to render them inactive. Adequate dilution will be secured if the amount of blood added to each bottle is limited to 5 ml. Mere dilution may not suffice if the patient was under treatment with sulphonamides or penicillin. The inhibitory effect of these may be overcome by adding to the broth *para*-aminobenzoic acid or penicillinase respectively. The skin of the antecubital fossa is treated with tincture of iodine and a tourniquet applied above this. The patient's arm is

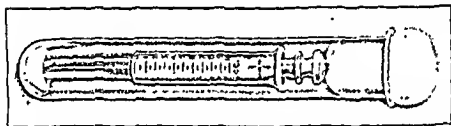


FIG. 35 — ALL-GLASS SYRINGE STERILISED IN LARGE TEST TUBE, READY FOR PERFORMING A BLOOD CULTURE.

extended and, if no veins are prominent, it is allowed to hang down for a few moments. The syringe is withdrawn from the tube and the stylette removed from the needle. The skin is stretched tightly to one side of the selected vein and the needle entered first through the skin, passed on a short distance in the subcutaneous tissue, and then into the vein. The left hand which stretched the skin is now transferred to the syringe and the right slowly draws out the plunger, filling the syringe with blood. When full, the tourniquet is removed and the needle sharply withdrawn. If the puncture has been properly made there will be no bleeding, owing to the valvular nature of the opening; but if a few drops of blood ooze out, tight pressure on the spot with a piece of cotton wool will quickly cause the flow to cease. The thread projecting from under

the cap of the bottle is pulled, so cutting the cap and usually causing it to fall off. This exposes the rubber (which is sterile) in the centre of the metal cap. The rubber is pierced with the needle and 10 ml. of blood injected into the bottle from the syringe or, preferable, 5 ml. into each of two bottles. When the needle is withdrawn the minute hole in the rubber seals itself. The bottle is shaken to diffuse the blood through the medium and is then incubated.

Occasionally it is of value in prognosis to carry out a quantita-

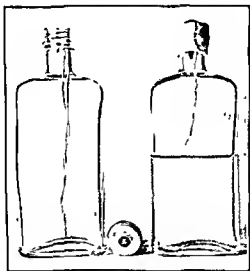


FIG. 36—BLOOD CULTURE BOTTLE

tive blood culture, that is a culture in which the number of bacteria present in the circulating blood is ascertained. This can be done by transferring a few millilitres of blood from the syringe to a sterile tube containing crystals of potassium oxalate to prevent clotting. A measured volume of this oxalated blood (say 1 ml.) is added to an agar stab, melted and cooled to 50° and, after mixing, the agar is poured into a plate which is incubated. From the number of colonies which develop and the volume of the blood inoculated, the number of bacteria per ml. can easily be calculated.

After incubation, the broth is examined microscopically and subcultures made on blood-agar or other medium. Blood cultures should be examined in this way at the end of 24 hours' incubation and then every 48 hours for a week. Where the presence of *Br. abortus* is suspected, blood cultures should not be discarded as sterile until incubation (in 10 per cent. CO_2) has been continued for 7 days. It is not believed that bacteria may be present in the blood.

Enteric bacilli may frequently be isolated from the clot left after the serum is removed from a specimen of blood submitted for the Widal test. The clot should be incubated in a large volume of bile salt broth.

Collecting Serum for Wassermann Tests, Agglutination, etc.

An all-glass or a Record syringe is sterilised by boiling either in water or in saline. If in water, it is essential to work the plunger gently up and down while the syringe is still hot, in order to expel all water which would cause hæmolysis. 10 ml. of blood are collected, as described in the technique of blood culture, and are expelled into a dry sterile tube. All bubbling and frothing must be avoided and, if some air is present with the blood in the syringe, it must not be expelled with the blood into the tube. The tube should be allowed to stand without agitation until clotting has taken place, when the clot may be gently loosened from the sides of the tube with a sterile wire or fine glass rod. It will then contract and, if the tube is left at room temperature until the next day, the clot will be found to have shrunk and to be covered and surrounded by clear serum free from red blood cells. This may be removed to a fresh tube by means of a sterile capillary pipette. The serum should be of a light yellow colour; if reddish, hæmolysis has occurred, either owing to the accidental addition of water, alcohol or other substance, to rough handling, or to bacterial contamination. If the serum is required quickly, the tube should be placed for 2 to 3 hours in the incubator or water bath at 37° .

At that temperature coagulation occurs rapidly and the clot quickly expresses the serum. At the end of that time the serum which surrounds the clot and which contains red blood corpuscles is pipetted off and the red cells deposited by centrifuging, when the clear supernatant serum can be removed. In taking blood for any serological test it is advisable to avoid the few hours following a meal, as at this time the serum may be heavily loaded with fat which may interfere with the test.

The Behring venule affords a very convenient method of collecting blood for serological tests. It consists of an evacuated receiving tube with a rubber stopper, through which passes a fine glass tube to the end of which a needle is fused. The fine tube is so arranged that its inner end is in contact with the rubber of the stopper, so forming a valve which prevents entry of air into the receiving tube unless the projecting fine glass tube is bent in relation to the rubber stopper. The needle is protected with a covering glass tube, and the whole is sterile. For use, the tube

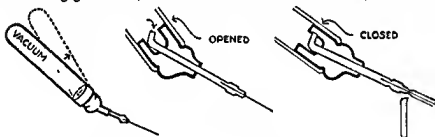


FIG. 37.—BEHRING VENULE.

protecting the needle is filed and removed, and the needle introduced into a vein. The connecting tube is then bent on the stopper and blood enters and fills the receiving tube. For transmission the needle may be removed and the outfit sent through the post.

Tissues and Organs obtained at Operation or Post-mortem Examination

Material such as this is very liable to have its exterior contaminated, and so it is necessary to obtain material for culture

from the interior. The best method is to sear an area with the cautery or a soldering-iron. This can be cut through with a sterile knife and material taken from the interior, or the seared part may be punctured with a sharp sterile capillary pipette and a few drops of fluid aspirated. In this way material for culture may be obtained from solid organs such as the spleen, or from the heart either of man or animal at post-mortem examination.

CHAPTER IX

THE IDENTIFICATION OF PATHOGENIC BACTERIA

It is impossible in bacteriology to devise a definite scheme of tests, the careful application of which will inevitably lead us to the correct answer to the question "What is this organism?" Nevertheless we should endeavour to work in as logical a manner as possible. The first step is a careful examination of the material in which the organism occurs, and in this connection the bacteriologist must, early in his career, learn to use some, if not all, his senses. The employment of the eyes is essential and of the sense of touch, through the intermediary of the platinum wire, the slide, or other implement, useful. Even the nose is by no means to be despised. In connection with the use of vision do not let us, because we deal with very minute objects, despise the naked eye, the hand lens or the low power of the microscope. The material should be thoroughly examined, for frequently from the colour, consistency or smell of pus, or from the turbidity of urine or cerebro-spinal fluid, we may derive much valuable information. Direct examination of a specimen of pus with the naked eye and with the hand lens may save us from overlooking the presence of the granules of actinomyces, which we may ignore if we merely make films and cultures from unselected parts. In the case of sputum the examination of carefully selected portions is very much more valuable than the preparations of films or cultures from random samples. The colour of pus may suggest such bacteria as the *Ps. pyocyanea*, and its smell may direct our attention to certain anaerobic bacteria.

Microscopic Examination

After these preliminary observations we may proceed to the use of the microscope. An unstained wet preparation may frequently be of assistance, particularly in the case of such fluids as urine,

since by its help we may ascertain the presence or absence of pus cells or other leucocytes and of red blood corpuscles. In dysentery, this examination will, in a high proportion of cases, enable us to say whether the disease is of amoebic or bacillary type. In many cases we may see in this preparation the bacteria themselves; we may be able to determine their morphology, whether bacilli or cocci and, if the former, whether motile, if the latter, whether streptococci or staphylococci or of uncertain grouping. In some cases, as in syphilis, it is possible to make a definite diagnosis of the disease from unstained films examined by dark ground illumination.

In the examination of some material (feces, for example, for the presence of bacilli of the enteric or dysentery groups) the examination of the bacteria in stained films does not yield any information of value; but in the great majority of cases films should be prepared, suitably stained and examined before cultures are made, as our cultural methods depend to a considerable extent on the variety of bacteria present. Simple stains are capable of furnishing a certain amount of information as to the morphology of the bacteria present; but Gram's method should also be used as a routine, as by its help we are able to classify the majority of bacteria into two groups—the Gram positive and the Gram negative. The pathogenic cocci, except the gonococcus, meningococcus and *N. catarrhalis*, are Gram positive. Of the bacilli, *C. diphtheriae*, *B. anthracis*, *Myco. tuberculosis*, *Myco. leprae* and the anaerobic bacilli are Gram positive; most other pathogenic bacilli are Gram negative. *V. cholerae* and all spirochaetes capable of being stained with simple stains are Gram negative.

Certain bacteria do not stain well, if at all, by either the stain or the counter-stain used in Gram's method. In this class we may place, as of greatest importance, *Myco. tuberculosis*, many of the other acid-fast bacilli and the majority of the spirochaetes. In examining pus, for example, when simple stains and Gram's stain do not reveal any bacteria, it is well to stain a film by the Ziehl-Neelsen method. The presence of spirochaetes is frequently first suspected on clinical grounds, and the special methods, such as

Fontana's, are not usually employed, except when we are expressly asked to ascertain the presence or absence of these organisms. Protozoa in the blood are usually discovered by direct microscopic examination of films, stained with Leishman's stain. In some cases special staining methods, such as that to demonstrate capsules, may be employed on films made from the material under examination.

We may now summarise the information which we can obtain by the microscopic examination of bacteria in preparations made either from the original material or from cultures. The size, shape and arrangement of the bacteria are first observed. The relatively large *B. anthracis* can hardly be confused with the much smaller *Bact. coli* or the minute *H. influenza*. The short oval of *Past. pestis* contrasts with the elongated rectangle of *B. anthracis*. *Bact. coli* is usually found as single elements, *B. anthracis* in chains and *C. diphtheria* in characteristic clusters. Unstained preparations help to differentiate *Salm. typhi* from *Sh. flexneri* on account of the motility of the former. The presence of a capsule differentiates *Bact. pneumoniae* from *Bact. coli*. The presence or absence of spores and, if present, their shape, size and position are of great assistance in identification. The large spherical and terminal spore of *Cl. tetani*, for example, is very characteristic. The importance of Gram's stain has already been mentioned, but other differential staining methods, especially that of Ziehl and Neelsen, by which, in the majority of cases, the tubercle bacillus is identified, are also of real service. Finally, the regularity or otherwise of staining of the bacterial cytoplasm—whether beaded, as in *C. diphtheria*, or chiefly polar, as in *Past. pestis*, is, in certain cases, of considerable help.

Cultural Examination

In some cases (e.g. tuberculosis, gonorrhœa, Vincent's angina) these preliminary examinations will suffice for the identification of the organism for purposes of diagnosis. In general, however, cultures are essential. The success of a culture depends to a considerable extent on the freshness of the material when cultured

and the suitability of the cultural methods. As a routine medium for the cultivation of the organisms causing disease in man, blood-agar may be used. Where the clinical history of the case, the source of the material, or the preliminary examination, points to a certain organism or group of organisms, special media or methods may be employed, such as desoxycholate-citrate or Wilson and Blair's media for intestinal bacteria, solidified serum for diphtheria bacilli and minced meat broth for anaerobic bacteria.

Incubation for 24 to 48 hours is, in general, sufficient. The plates or other cultures should be examined and, if the former have been properly prepared, well-isolated colonies should be seen. The examination of colonies with the naked eye, hand lens or low power of the microscope as well as the consistency of the colony, as judged by touching with a platinum wire, are of the highest importance in identifying bacteria. Such points as the colour, size, shape, outline (whether even, crenated or irregular), surface (whether shiny, matt or rough), whether transparent or opaque, whether elevated, flat or depressed in the centre and whether dry or moist, should all be carefully noted. It should be observed whether the colony is sticky, tough, or lumpy and difficult to break up, and whether it is easily removed or adherent to the medium.

If blood-agar is employed, any alteration produced in the medium is of great importance. Some bacteria cause hæmolysis of the blood cells distributed through the agar owing to the diffusion of a hæmolysin. The result is that the plate shows a zone of clearing around the colony, but this zone is still of a blood-red colour. In what is commonly called hæmolysis, however, something more than mere laking of the cells has occurred, as the zone not only becomes clear, but also loses almost all its red colour. Some of the hæmoglobin has been absorbed into the colony, some has diffused into the surrounding medium, but a considerable part has been altered into a colourless compound. On blood agar some bacteria (e.g. pneumococci) produce a green coloration due to a hæmoglobin derivative of undetermined nature.

While the appearance of the colony is an important characteristic of an organism, wide variations from the normal are sometimes observed. In the case of the intestinal bacilli the normal type of colony has an even outline and a smooth shining surface, but variants, in which the outline is crenated and the surface irregular and rough, are sometimes obtained by plating material from the body of a patient. . . .

staphylococci are seen in fig. 38. It has been observed that while smooth, normal colonies form a uniform stable suspension in saline, the rough types form suspensions which are granular and spontaneously agglutinating, although stable suspensions may



FIG. 38.—VARIANT COLONIES OF *Staphylococcus pyogenes*.

usually be obtained by decreasing the strength of salt in the solution. We shall have occasion later to refer to antigenic differences and to alterations in virulence which occur when variant colonies are produced.

When the colonies on the plate have been carefully examined, films of each variety should be prepared and stained by Gram's method. After this, it may be possible to state definitely what the organism is; but if several types of colonies are present it is usual to obtain each variety of organism in pure culture before further investigations are carried out. In some instances it is useful to contrast the morphology of the organism when grown on different media (streptococci usually produce longer chains in fluids than on solids). The presence of spores should be investigated in

suitably stained films. The temperature of growth (maximum, optimum, or cus, for exam of the body, Another poi

growth is obtained, such as whether the organism will grow on plain agar or demands an enriched medium, and whether it grows only or better under aerobic or anaerobic conditions. The naked

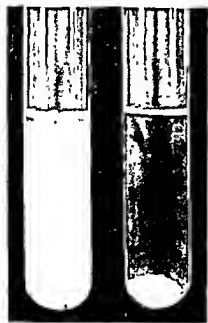


FIG. 39.—ON LEFT, UNIFORM TURBIDITY OF *Salm. typhi* IN BROTH. ON RIGHT, DEPOSIT OF STREPTOCOCCI IN BROTH

Salm. typhi and the deposit of streptococci in broth culture are suggestive indications of the organism in the culture. The microscopic examination of a broth culture is the most reliable method of determining whether or not a bacterium is motile.

Biochemical Characteristics

Gelatin cultures are useful in showing us whether the bacteria we are investigating liquefy that substance or not. *V. cholera*, *B. anthracis*, staphylococci, and *Proteus vulgaris* cause liquefaction: streptococci, *Bact. coli*, and the

enteric and dysentery bacilli do not.

The products of growth are also utilised in the identification of bacteria. Among these may be mentioned the production of indole in broth or peptone water. This may be tested for by the Nitroso-indole method: to the broth culture are added a few drops of a 0.1 per cent. solution of potassium nitrite followed by sul-

phuric acid, when a rose colour indicates the presence of indole. A more sensitive test is Ehrlich's Rosindol Reaction. Ehrlich's reagent is carefully added to the broth with a pipette so that it floats on the surface, and the tube is gently agitated to secure a slight mixing between the fluids. If indole is present, a red ring develops and the colour diffuses through the supernatant solution.



FIG 40 —LIQUEFACTION OF GELATIN BY *Staphylococcus pyogenes*

any strains of *Sh. flexneri* from *Salm. typhi* which in other respects behave similarly. If, in addition to indole, nitrites are also produced, the nitroso-indole reaction is given on the addition of sulphuric acid alone. This is known as the "Cholera Red Reaction", and distinguishes *V. cholerae* from many other vibrios which it otherwise resembles.

The Voges-Proskauer reaction is useful in distinguishing certain members of the group of intestinal bacilli. The test is carried out by growing the organism for 1 or 2 days in the buffered glucose broth described in Chapter XLVIII. Incubation at 30° is more satisfactory for this reaction than at the customary 37°. To the culture is added a knife point of creatin (as recommended by O'Meara) and an equal volume of 40 per cent. sodium hydrate solution. The whole is vigorously shaken and, if the test be positive, a red colour develops in a few minutes.

A positive reaction is due to the production of

acetyl-methyl-carbinol. A satisfactory alternative method of demonstrating the production of acetyl-methyl-carbinol is that of Barritt. To 10 ml. of a culture in buffered glucose broth are added 0.6 ml. of a 5 per cent. solution of α -naphthol in alcohol and 0.2 ml. of a 40 per cent. solution of potassium hydroxide. The tube is vigorously shaken and, if the result is positive, the fluid

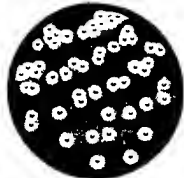
becomes pink in a few minutes, the colour deepening to crimson or magenta within 1 hour.

The fermentative activity of an organism is, in the case of the members of some groups, of outstanding importance for identification. To suitable medium (peptone water most usually) 0.5 to 1.0 per cent. of various carbohydrates are added and the medium then used for the cultivation of the organism. It may either fail to act on the carbohydrate or it may ferment it, producing either acid alone or gas as well as acid. The production of acid may be determined by the addition to the medium of an indicator which changes colour when acid is produced. Gas, if any is evolved, collects in a small test tube inverted in the medium. The gas is generally a mixture of hydrogen and carbon dioxide and the proportion of the two may be of some importance. Gas production may also be judged by the 'splitting' caused in solid media containing fermentable carbohydrates, and when the amount of gas is small this method is more delicate than that in which fluid media are used. The determination of the fermentative activities of the organism is most useful in the case of the Gram negative intestinal bacilli in which morphology assists very little in identification.

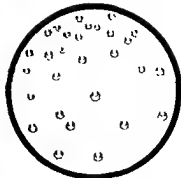
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 methyl-red solution are added and the resulting colour noted. If red (showing high and persisting acidity), the test is positive; if yellow (indicating low or transient acidity), it is negative. This test is chiefly used for distinguishing coliform bacilli isolated from water.

Serological Methods of Identification

As a result of the various procedures described we are, at this stage of the investigation, either quite satisfied as to the identity of the organism or have narrowed the choice down to two or three; the next appeal is frequently to serology. When an animal, such as a rabbit, is inoculated with an organism, antibodies are



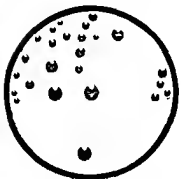
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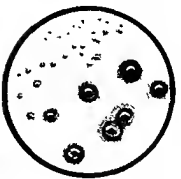
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5



3



6

COLONIES OF BACTERIA

- | | |
|---|--|
| 1 Streptococci on blood agar showing α <u>h</u> emolysis | 4 <i>Salmonella typhi</i> on MacConkey's medium |
| 2 Streptococci on blood agar showing β <u>h</u> emolysis. | 5 <i>Bacterium coli</i> on MacConkey's medium |
| 3 <i>Staphylococcus pyogenes</i> on blood agar. | 6 <i>Salmonella typhi</i> on Wilson and Blair's medium |

becomes pink in a few minutes, the colour deepening to crimson or magenta within 1 hour.

The fermentative activity of an organism is, in the case of the members of some groups, of outstanding importance for identification. To suitable medium (peptone water most usually) 0.5 to 1.0 per cent. of various carbohydrates are added and the medium then used for the cultivation of the organism. It may either fail to act on the carbohydrate or it may ferment it, producing either acid alone or gas as well as acid. The production of acid may be determined by the addition to the medium of an indicator which changes colour when acid is produced. Gas, if any is evolved, collects in a small test tube inverted in the medium. The gas is generally a mixture of hydrogen and carbon dioxide and the proportion of the two may be of some importance. Gas production may also be judged by the 'splitting' caused in solid media containing fermentable carbohydrates, and when the amount of gas is small this method is more delicate than that in which fluid media are used. The determination of the fermentative activities of the organism is most useful in the case of the Gram negative intestinal bacilli in which morphology assists very little in identification.

The methyl-red test depends on the final reaction of the medium after glucose fermentation. Buffered glucose broth is inoculated with the organism and incubated for 4 days. A few drops of methyl-red solution are added and the resulting colour noted. If red (showing high and persisting acidity), the test is positive; if yellow (indicating low or transient acidity), it is negative. This test is chiefly used for distinguishing coliform bacilli isolated from water.

Serological Methods of Identification

As a result of the various procedures described we are, at this stage of the investigation, either quite satisfied as to the identity of the organism or have narrowed the choice down to two or three; the next appeal is frequently to serology. When an animal, such as a rabbit, is inoculated with an organism, antibodies are

produced to that organism and are found in the animal's serum.

of being *Salm. typhi*, is agglutinated by an anti-typhoid serum, we can usually decide definitely that the bacillus is *Salm. typhi*. The macroscopic method should be used, and the unknown organism should be agglutinated to at least one-third the titre of the serum for its homologous organism. In some cases simple agglutination may be insufficient agglutinins in the serum, a "absorption of agglutinin" described elsewhere.

Pathogenicity

The final test to be applied for the identification of certain organisms is that of animal inoculation. Many bacteria which are pathogenic for man are also pathogenic for animals, and the results of infection with certain organisms, as seen at a post-mortem examination, may be sufficiently characteristic to make the presumptive identification certain. This is true, for example, of the tubercle bacillus and the anthrax bacillus. Those bacteria which produce a powerful exotoxin may be identified by finding that their cultures produce a characteristically fatal result in one animal but not in another which has been injected simultaneously with antitoxic serum. This is the method used to prove that an organism resembling the *C. diphtheriae* in morphology and cultural characteristics is actually a virulent *C. diphtheriae*.

It is not necessary to employ all the methods described for every bacterium which it is required to identify. In the case of some bacteria it may be possible to name the unknown organism definitely in a day or two, in the case of others this may occupy a matter of weeks or even months. It is only by a fairly complete knowledge of the characteristics of the various types of bacteria, of the many cultural methods and of the niceties of technique,



diagnosis or treatment, to know whether a patient has been infected with a type A or a type B typhoid bacillus; but this knowledge is of great value in tracking an epidemic to its source. If, for example, several cases of typhoid fever in a community are found to be due to one type, and several to another, it is at once obvious that the ultimate sources of infection are different in the two groups. By an intensive search for carriers and by typing the bacilli excreted by them, responsibility for originating an epidemic may often be attached, with almost complete certainty, to individual carriers.

In the case of *C. diphtheriae* it is possible, on a basis of morphology, cultural characteristics and pathogenic powers, to distinguish three varieties which are usually described as types—gravis, intermedius and mitis. The differences between the three are much more marked than are those used in some genera to differentiate species, but since they produce the same toxin and the same disease we are unwilling to allot them to different species. We propose, therefore, to refer to them as the three groups of *C. diphtheriae*. A number of types can be differentiated within each group by serological methods.

Koch's Postulates

Under what conditions are we able to state positively that a given organism is the cause of a certain disease? The most strict criteria are those known as Koch's Postulates.

1. The organism should be present in the tissues or fluids of the affected animal.

2. It should be isolated and cultivated outside the body for several generations.

3. The cultivated organism, on inoculation into a suitable animal, should reproduce the disease.

4. It should be again isolated from the artificially infected animal.

It is not possible in every case to fulfil all these requirements, as, for example, in the case of those viruses which cannot be cultured and of those bacteria which are pathogenic for human beings but

that the bacteriologist is able to select the methods of investigation necessary in each case and to avoid those not likely to yield useful information.

Differentiation of Types

It is no longer, in all cases, considered sufficient to determine the genus and species to which a given organism belongs. Now we frequently require, in addition, to know its type within the species.

The criteria on which the determination of types depends vary with the species. The most commonly used basis of type differentiation is differences in the chemical composition of the bacteria themselves, their appendages (flagella or capsules) or soluble products. Since the detection of these differences by chemical means would often be quite impossible, we utilise the living animal body to do what the chemist cannot. Many of these chemical substances are antigenic, that is, when introduced into the animal body, they stimulate the formation of antibodies which react with a high degree of specificity with the corresponding antigen. The differentiation may be applied to somatic antigens (e.g. in streptococci), to capsular antigens (e.g. in pneumococci) or to toxic antigens (e.g. in *Cl. perfringens* and *Cl. botulinum*).

A completely different method which, however, has probably also a chemical basis, is used for the division into types of *Salm. typhi*. Viruses, known as bacteriophages or phages, exist which are capable of dissolving or lysing these bacteria. Some of these phages are highly specific, acting even in high dilution on one strain and either not acting at all or acting only when concentrated on another. Among these phages are some which can be conditioned or trained to act on a strain against which they were previously relatively inactive. All strains of the bacterium which are lysed by a particular conditioned phage are placed in the same type to which a letter (A, B, C, etc.) is arbitrarily allotted. Similar methods have been applied to the typing of *Staph. pyogenes* and there is little doubt that the method will ultimately be used for typing other species of bacteria. It is of no importance, either for

diagnosis or treatment, to know whether a patient has been infected with a type A or a type B typhoid bacillus; but this knowledge is of great value in tracking an epidemic to its source. If, for example, several cases of typhoid fever in a community are found to be due to one type, and several to another, it is at once obvious that the ultimate sources of infection are different in the two groups. By an intensive search for carriers and by typing the bacilli excreted by them, responsibility for originating an epidemic may often be attached, with almost complete certainty, to individual carriers.

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not for any of the lower animals, but they represent the ideal to be aimed at. Since the postulates were formulated, immunology has given us some useful methods which help us to connect an organism with a disease, and every bacteriologist is satisfied that the causative organisms of several diseases are known although all the postulates have not been satisfied.

CHAPTER X

ANTISEPTICS, CHEMOTHERAPY, ANTIBIOTICS

Certain chemical substances either kill bacteria or prevent their growth in the presence of nutrient material. Those which kill bacteria are described as bactericidal, those which prevent bacteria growing without killing them as bacteriostatic. It is not always an easy matter to determine to which class a particular substance should be assigned as most substances which are bactericidal in high concentration are bacteriostatic in low. The converse is not always true: some substances, even in the highest attainable concentration, are only bacteriostatic. A further difficulty is that, if bacteria are prevented from growing, they have a tendency to die, so that it is a matter for philosophers rather than bacteriologists to decide whether the bacteria were killed by the substance or, in its presence, died.

The terms most commonly applied to antibacterial substances are antiseptic, disinfectant and germicide.

An antiseptic is, as its name implies, a substance which prevents putrefaction or sepsis, but the word has acquired a wider meaning and may now be applied to any substance which prevents the growth of bacteria. An antiseptic may be either bactericidal or bacteriostatic in its action.

A germicide is a substance which kills germs, that is, bacteria. It is obviously a bactericidal substance.

A disinfectant is a substance which prevents the spread of infectious disease by killing the causative organism of the disease. A substance which kills pathogenic bacteria is certain to kill other types as well, but the term does not imply that the substance is capable of sterilising, that is of killing all forms of bacterial life. A disinfectant is, therefore, bactericidal, but possibly only to a limited extent.

It is a good practice to use the term antiseptic when the precise mode of action of the substance under consideration is either uncertain or unimportant, and the term bactericide and bacteriostat when we wish to be specific as regards the action of the substance.

Many substances act as antiseptics—acids and alkalies, oxidising and reducing substances, halogens, metallic salts, alcohols, ethers and aldehydes, volatile oils and aniline dyes. While their precise mode of action differs, the end result of those which are bactericidal is so to alter the bacterial protoplasm as to render it incapable of behaving as a living substance. In other words, these antiseptics are protoplasmic poisons from which it follows that, while they kill bacteria, they also kill or injure other cells, including those of the human body. Most antiseptics are, to a greater or less extent, selective. That is, they act in higher dilution against some bacterial species than against others. With a few, selectivity goes further than this and those which are more injurious to bacterial protoplasm than to the protoplasm of mammalian cell, are of especial value in the treatment of tissues invaded by bacteria.

In order to kill bacteria, an antiseptic must be brought into intimate association with them. For this reason most antiseptics are effective only when in solution. Some, however, are equally, or even more, effective when applied in the form of emulsions.

In assessing the value of an antiseptic as a bactericide the following factors must be considered—1. Nature of the bacteria; 2. Number of bacteria; 3. Concentration of the substance; 4. Temperature; 5. Rate of killing; 6. Environment

Each antiseptic has its own peculiarities as regards the species of bacteria against which it acts best. The underlying principles are almost completely unknown and actual experiment is required to determine whether a given substance is of value against a particular species. The aniline dyes are mostly highly specific and use is made of this peculiarity by incorporating them in culture media which, as a result, permit the growth of certain bacteria while completely suppressing the growth of others. Almost the only generalisations which it is safe to make are that spores are

more difficult to kill than are vegetative forms and that bacteria with a high lipid content, such as the tubercle bacillus, are more resistant than other non-spore-forming organisms.

The number of bacteria which are acted upon by an antiseptic does not significantly affect the potency of a particular concentration unless the number is very large and the antiseptic is one which, in whole or part, enters into chemical combination with bacterial protoplasm (e.g. the mercurials, oxidising agents).

The next three factors, concentration, temperature and rate of killing, are so interdependent that they must be considered together. Safe generalisations are that bacteria are more rapidly killed by high concentrations than by low and at high temperatures than at low. Each antiseptic has its own characteristics as regards the rate at which it kills bacteria and the effects of concentration and temperature. Some antiseptics, particularly the halogens, are rapid in action; some, such as salts of mercury, slow. Increasing the concentration of the antiseptic has a much more marked effect in accelerating killing with some antiseptics than others. Doubling the concentration of phenol increases its rate of action sixty-four times, while a similar increase in the concentration of mercuric chloride only doubles its rate of killing.

A rise in the temperature at which an antiseptic acts on bacteria has a more marked effect in some cases than in others. A rise of 10° increases the rate of killing of bacteria by a particular concentration of phenol eight times: the same rise increases the rapidity of action of mercuric chloride two or three times.

All the individuals in a bacterial population are not killed by an antiseptic at exactly the same moment. Although the majority are killed within a very short time of one another, some are killed more rapidly and some more slowly.

In evaluating an antiseptic it is customary to determine, for a particular concentration acting on an ascertained number of bacteria of known species, the time required to sterilise, that is to kill all the bacteria in a measured volume of the test fluid. This gives rather a wrong impression of the potency of the antiseptic since the time required to kill the last 1 per cent. of the bacteria

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has a high Rideal-Walker coefficient may be almost useless as a disinfectant of the secretions or excretions of a patient. To meet these criticisms, various modifications have been introduced. In the best of these, the Chick-Martin test, dried sterilised faeces are added to the tubes and the time of action is fixed at 30 minutes: the test organism is, as in the Rideal-Walker method, the typhoid bacillus. This modification does not meet the criticism that the test uses the time required to sterilise rather than that necessary to kill 50 per cent. of the bacteria.

There is no ideal or universal disinfectant. We must select the one which best serves our particular purpose. There is little probability of the substance considered most useful for the disinfection of the faeces of a typhoid fever patient being best for the preparation of the skin prior to operation or for the irrigation of an infected bladder.

For the disinfection of faeces, urine and sputum, Lysol (cresols dissolved in soap solution) or one of the many other coal-tar disinfectants (Izal, Cyllin etc.) in which the cresols are held in suspension as emulsions by gums or oils, are commonly used. These disinfectants, which are cheap, are reliable because the cresols are not rendered inert by the presence of proteins.

Chlorine and certain compounds the active constituent of which is chlorine are unequalled for the disinfection of water, but are almost useless, except in great excess, for disinfection of secretions and excretions containing much protein material.

Dettol, the active constituent of which is xyleneol modified by the addition to its molecule of an atom of chlorine, is better tolerated by the tissues than the majority of active disinfectants. It has, as a further advantage, the fact that its potency is but little reduced in the presence of blood and pus.

For the disinfection of the skin many antiseptics, including such dyes as gentian violet, have been used, but it is doubtful if any is superior to iodine. A solution of 2.5 per cent. iodine and 2.5 per cent. potassium iodide in 90 per cent. alcohol is effective and not excessively irritating. Alcohol itself has some disinfecting power on which, however, too much reliance should not be placed.

may be double that which suffices to kill 99 per cent. For this reason it is preferable to determine for, a particular concentration, the LD 50, that is the time required to kill 50 per cent. of the bacteria present.

The Rideal-Walker test has been very extensively used to assess the potency of disinfectants. In this test, tubes containing various dilutions in water of phenol and of the antiseptic under test are inoculated with *Salmonella typhi* and subcultured into broth after 2½, 5, 7½, 10, 12½ and 15 minutes. The broths, after incubation, show whether the bacteria survived or not and so we learn, for each concentration of each antiseptic, the time required to sterilise the volume tested—one loopful. The results are set out in tabular form and a dilution of the new antiseptic which gives exactly the same results as one of phenol is selected. Suppose that the 1 : 100 dilution of phenol does not kill all the typhoid bacilli in 2½ or 5 minutes but does in 7½ minutes and that the 1 : 350 dilution of the antiseptic under test gives exactly the same result, the carbolic acid (or Rideal-Walker) coefficient of the antiseptic is $350 \div 100 = 3.5$. In other words, the antiseptic is, as judged by this method, 3.5 times as powerful as phenol.

Various criticisms of the method have been made. One is that the test organism is the typhoid bacillus, while the disinfectant may be required to combat other pathogenic bacteria, the resistance of which may be very different from that of the typhoid bacillus. Another criticism is that the method yields a higher coefficient for disinfectants which act rapidly than it does for more slowly acting substances. In this connection it may be said that mercuric chloride has a Rideal-Walker coefficient of 2 (that is, it is twice as powerful as phenol), when the test period is 2½ minutes while, when the test period is 30 minutes, its coefficient is over 500. The chief criticism of the method, however, is that it tests the efficacy of a disinfectant in an environment consisting mainly of water and almost devoid of protein. In practice we require of a disinfectant that it shall, within a reasonable time, kill pathogenic bacteria in the presence of blood, pus, sputum or faeces, that is, in a richly protein environment. A substance which

bacteria. In the absence of these cells, and protected by the necrotic material composed of other dead cells, any surviving bacteria and those subsequently introduced flourished and sepsis persisted.

Fleming, in one of his elegant experiments, showed the effects of varying concentrations of phenol in a wound. He made a series of dilutions of phenol in blood inoculated with streptococci. Each preparation contained the same amount of blood, complete with its plasma, red cells and white cells, and the same number of streptococci. The only variable was the phenol which was present in highest concentration in the first preparation in the series from which the amount fell to the last which contained none. In the preparations containing the highest concentrations of phenol, there was no evidence of growth of the streptococci. This was followed by a zone in which the streptococci had survived and grown. In the later preparations, as also in the control which contained no phenol, there was again no evidence of growth of the streptococci. The explanation of these results was that, in high concentrations of phenol, both the bacteria and the leucocytes had been killed, in lower concentrations the leucocytes but not the streptococci had been killed and the latter had grown, unrestrained by the small amount of phenol present. In the final preparations, neither the streptococci nor the leucocytes had been killed by the phenol, but the leucocytes had ingested and destroyed the bacteria. The lessons to be learned from this experiment are that the leucocytes, on which the body mainly depends for its defence, are more easily killed by crude antiseptic substances than are the bacteria and that the application of such substances to wounds may do more harm than good.

Antiseptic surgery gradually gave place to aseptic in which heat is used for sterilisation of instruments, etc., and no injurious materials are brought into contact with exposed tissues.

Many new antiseptics have been introduced since Lister's time and some of these are so selective in their action that it is possible to use them in a concentration at which they are lethal for at least some species of bacteria but not for tissue cells or phago-

Alcohol diluted with water (50 to 70 per cent. alcohol) is more effective than is absolute alcohol.

For protection against bacterial contamination of biological products such as sera and vaccines intended for injection, the antiseptic selected must not, in the dose introduced, be toxic, must not give rise to severe irritation and must not cause precipitation or other alteration in the product. Phenol, formalin, alcohol, glycerol, merthiolate (an organic mercurial salt) and many others have been used for this purpose.

The discovery that it is possible to kill bacteria suspended in the air by finely dispersed chemical substances is a relatively recent one. Conditions here are completely different from those found in fluids and some of the substances most effective for the disinfection of the air are relatively weak in a fluid medium. Among substances used for this purpose, in addition to such ordinarily recognised disinfectants as hypochlorites, are lactic acid and propylene glycol. The latter, when vapourised, kills practically all bacteria in air within 5 minutes when acting in a concentration of one part in two and a half million parts of air.

Chemotherapy

Lister, appreciating the importance of Pasteur's discoveries in bacteriology and convinced that sepsis was due to the introduction into the tissues of bacteria, devised his antiseptic method of surgery. He relied on the bactericidal power of carbolic acid and other chemical substances to kill bacteria on the hands, instruments and dressings of the surgeon and in this way rendered it possible to secure the healing of wounds without the sepsis which, previously, had been almost universally present. He did not hesitate to introduce antiseptics into wounds where they were in immediate contact with unprotected tissues. It was not long before it was realised that antiseptics in a wound were rarely beneficial and often harmful. In the presence of established sepsis, they usually failed to sterilise the wound, that is to kill all the bacteria in it, but they did kill large numbers of cells, including the phagocytic cells which are the body's chief defenders against invading

disadvantages of the various sulphonamides. Some are more successful against one organism, some against another. some are specially indicated for a particular purpose because they are fairly soluble, others, for another purpose, because they are but slightly soluble; some are of high efficiency but are toxic, others of less efficiency are to be preferred because they are little liable to give rise to toxic symptoms.

The outstanding features of the sulphonamides are that they are bacteriostatic and, to some extent, bactericidal in low concentrations (from $1 : 100,000$ to $1 : 10,000$), that in such concentrations they are harmless to phagocytic and tissue cells, and that they can be administered orally.

There is no generally accepted view as to their mode of action but it may be taken as established that they do not act, as do the majority of ordinary antiseptics, by coagulating or otherwise crudely altering the chemical or physical nature of bacterial protoplasm. Rather do they appear to starve the bacteria by depriving them of some essential metabolite with the result that they cease to multiply and ultimately die. It is possible that, through similarity in chemical structure of sulphonamide and a substance required by the bacteria for synthesis into protoplasm or a precursor of such a substance, the limited amount of enzyme available for this synthesis is preoccupied by the sulphonamide and so is not available for its legitimate purpose. While there may be several such substances, there can be little doubt that one of them is *para*-aminobenzoic acid

As has been stated above, sulphonamides do not attack all species of bacteria. They are of chief value against the pathogenic cocci (except *Staphylococcus pyogenes* which is attacked only by some of them and by these only to a limited extent), many of the Gram negative bacilli and especially the shigellæ, and species of the genera *Corynebacterium*, *Hamophilus* and *Clostridium*.

Since not all strains of a bacterial species normally sensitive to sulphonamide action are sensitive, it is advisable, before treatment is instituted, to test the patient's strain. This can be done by one of the methods described elsewhere.

cytes. The acridines deserve special mention as they are of definite value in controlling, if not in overcoming, local infections. None, however, of the older antiseptics, when administered orally or by injection, is capable of combating deep seated infections.

Chemotherapy means treatment of a disease by a drug which has a lethal or inhibitory effect on the causative organism of the disease but which does not, in effective concentrations, inflict damage on the cells of the body. As has been explained above, there are a number of chemical substances which have a useful chemotherapeutic action locally but for many years only one drug—quinine—was successfully applied to the treatment of a disease, the causative organism of which was widespread throughout the body. The organism against which quinine is effective, the malaria parasite, is, of course, a protozoon and not a bacterium.

Ehrlich, the founder of chemotherapy, sought for a drug which, like a "magic bullet", would destroy in the body the pathogenic organisms at which it was aimed. He believed that the drug he sought would be a dye and so he evolved many new dyes, some of which were active against bacteria but only in concentrations which were toxic for the patient. His greatest discovery was Salvarsan, an organic arsenical compound which was not a dye. This was the precursor of a number of similar substances, widely used in the treatment of syphilis. In 1935, Domagk, still under the influence of Ehrlich, while investigating the chemotherapeutic properties of various dyes, discovered Prontosil. This is an azo-dye into the molecule of which a sulphonamide group had been introduced because it had long been known that this group increased the fastness of dyes for silk and wool. Within a short time, Prontosil was shown to be by far the most successful drug ever employed in the treatment of natural infections of man and experimental infections of animals.

Tréfouël soon showed that Prontosil was active against bacteria, not because it was a dye, but because in the body it was broken down to para-aminobenzene sulphonamide now known as sulphamylamide, the first of the many sulphonamides

It is not possible here to consider the relative advantages and

disadvantages of the various sulphonamides. Some are more successful against one organism, some against another; some are specially indicated for a particular purpose because they are fairly soluble; others, for another purpose, because they are but slightly soluble; some are of high efficiency but are toxic, others of less efficiency are to be preferred because they are little liable to give rise to toxic symptoms.

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Sulphonamide therapy may fail in the presence of large numbers of bacteria or of pus. So treatment of a patient with an abscess may not succeed until its contents have been evacuated.

Since sulphonamides are fairly rapidly excreted from the body, mostly in urine, it is necessary, in order to maintain an effective concentration, to administer an adequate dose every 4 hours or so. The oral route is satisfactory but, where it is of importance to get a high concentration as rapidly as possible, one of the more soluble sulphonamides may be introduced intravenously. The guiding principle in treatment should be high dosage for a short period rather than low dosage for a long period. If the treatment is going to succeed, there will be indications of a successful outcome within a few days. In the absence of such indications, the treatment should be abandoned. Such serious complications as agranulocytosis most commonly occur when treatment has been continued (often at a low level) for a long time. A serious objection to low dosage is the tendency of bacteria exposed to sub-inhibitory concentrations of sulphonamide to develop resistant forms. This acquired resistance persists for many generations, possibly indefinitely. It is because many strains of the gonococcus have acquired resistance to sulphonamides that these drugs are now less successful in the treatment of gonorrhœa than they were when first introduced.

Antibiotics

A substance produced by an organism which is antagonistic to the life or growth of another organism is called, not very happily we think, an antibiotic.

Pasteur was probably the first bacteriologist to observe that the proximity of a colony of one type of bacterium might prevent the growth of another type. Many others carried the investigation further and before the end of last century pyocyanase, a product of the growth of *Ps. pyocyanea*, had been obtained in reasonably pure form. Pyocyanase has the power of killing and of dissolving bacteria of several species, but it is too toxic for clinical use.

A number of workers carried on the study of antibiotics pro-

duced by bacteria, fungi and molds, but it was not until penicillin had, in clinical trials, demonstrated its value, that most workers realised the practical value of antibiotics.

Penicillin was discovered by Fleming in 1929, but it was almost completely neglected until 1941 when, largely as a result of the work of Florey and Chain, it became available in sufficient amount for clinical trial. Almost at once this product of the growth of a mold, *Penicillium notatum*, established itself in a position which it still holds as the greatest of the antibiotics.

Penicillin has been synthesised, but synthetic penicillin is and is likely to remain a chemical curiosity as extraction from the medium in which the mold has grown is a more economical method of production.

Penicillin, as produced naturally, is a mixture of four chemically distinct but closely related substances known by British workers as penicillins I, II, III and IV which correspond respectively to the American F, G, X and K. The four differ slightly in their potency against different organisms, their rate of destruction in and elimination from the body and in other respects, penicillin IV (K) being of least therapeutic value. It is possible, by suitable modifications in the method of manufacture, to secure a relatively high yield of one or more of these at the expense of the others and, during purification, to eliminate the least efficient one. The penicillins are complex organic acids which are usually supplied as salts of sodium or calcium. In this chapter we propose to ignore the minor differences of the four penicillins and consider them as one substance. Some brands of penicillin intended for clinical use are of a yellow colour, but pure penicillin is quite colourless.

The penicillin now available is much more stable than were the impure samples first used therapeutically. Kept dry and in sealed containers at air temperature, the loss in potency of penicillin is negligible over a period of several months. Solutions are much less stable and, if they are to be kept at all, should be stored at refrigerator temperature. They should be rigorously protected against bacterial contamination. Penicillin is destroyed by acids and alkalis and by many other chemical substances. For this

reason it should not be mixed with any other material until the latter has been found to be innocuous.

Several species of bacteria (*Ps. pyocyanea*, some of the coliform bacilli and some species of the genera *Proteus* and *Bacillus*), which are resistant to the action of penicillin, owe their resistance to the production of penicillinase, a substance, possibly an enzyme, which rapidly destroys penicillin. Penicillin may fail to overcome a highly susceptible organism if this is present in a lesion with other bacteria, some of which are penicillinase producers.

Penicillin is undoubtedly the most potent antibiotic or chemotherapeutic remedy we possess but it is not equally potent against all species of bacteria. It acts powerfully against almost all pathogenic cocci, Gram positive as well as Gram negative, including *Staph. pyogenes*. Among other susceptible organisms are *C. diphtheriae*, *B. anthracis*, some strains of *H. influenza*, *Tr. pallidum*, other spirochaetes and many of the clostridia. The majority of Gram negative bacilli are resistant although some (such as *Salmonella typhi*) are only moderately so.

Even among normally susceptible species, such as *Staph. pyogenes*, some strains are encountered the resistance of which is naturally much greater than that of normal strains and some which are almost insensitive. As a result of prolonged exposure to concentrations too weak to be even bacteriostatic, resistance to penicillin can be artificially induced. For these reasons, it is almost essential to test the sensitivity of the patient's own strain before penicillin therapy is instituted.

The action of penicillin is mainly bactericidal although, in sublethal concentrations, it is also bacteriostatic. About its mode of action there is still much which is obscure, but there can be no doubt that it acts in some way by interfering with bacterial metabolism. In order to kill, penicillin must be absorbed into the cell. This can occur only during active metabolism which is an essential preliminary to division, so bacteria, even of the most susceptible types, are unaffected by penicillin when suspended in a non-nutrient medium. When penicillin is absorbed, one result is interference with division so that giant forms are produced.

Some types of bacteria, killed by penicillin, dissolve. This is probably due to autolysis of dead bacteria rather than to any specific action of the antibiotic.

In the lowest concentrations with any demonstrable antibacterial effect, penicillin acts mainly as a bacteriostat. In slightly higher concentrations, it is bactericidal and, over a rather narrow range, increases in concentration increase its bactericidal activity. A concentration is soon reached, however, which has maximum lethal power. Even enormous further increases in concentration do not increase either the rate of killing or the number of bacteria killed.

Penicillin, despite its great lethal powers, does not rapidly sterilise a fairly dense suspension of bacteria. Almost, but not quite all, the bacteria are killed within 24 hours but, if the penicillin is destroyed, the survivors sooner or later multiply. The explanation of this phenomenon, which is of great practical importance, is that, in even the most actively growing culture of bacteria, there are a few dormant forms which have been termed persisters. Since these are not "feeding" they are unaffected by penicillin.

The duration of the period of dormancy varies greatly from individual to individual. In some it may last only a few hours, in others for days, in a few for more than a week. As soon as a dormant form resumes activity it absorbs penicillin and is killed. Penicillin in relation to persisters acts in the same way as a trained police dog in relation to a fugitive. So long as he remains quiet he is not attacked, if he displays any activity it attacks him.

Penicillin administered orally is absorbed from the intestinal tract but so much of it is destroyed by the acids and alkalis of the digestive system and by the penicillinase produced by intestinal bacteria that the oral method of administration is too grossly extravagant and unreliable to be practicable, except possibly in infants. Penicillin must, therefore (apart from local application to the skin, mucous membrane, cavities and wounds), be injected either intravenously, intramuscularly or subcutaneously. It is very rapidly absorbed and, within a few minutes of intramuscular injection

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the intrathecal space and in abscesses, may still be inadequate, it is often an advantage to supplement general methods of administration by injecting a solution containing say 5000 units per ml. directly into these cavities once every 24 or 48 hours.

Although the chemical formula of penicillin is now known, assay of a batch of penicillin by chemical means is too difficult to be practicable and so biological assay is usually employed. The dose is expressed in terms of units and not of weight. The international unit is based on the original Florey or Oxford unit which was arbitrarily adopted. It is an amount of penicillin which has the same antibacterial activity (against the "Oxford" strain of staphylococcus) as 0.0006 mg. of pure sodium penicillin II. To assay a batch of penicillin it is necessary to have a solution of standard penicillin, the strength of which in terms of international units is exactly known. Suitable dilutions of the two are tested for antibacterial activity against the same culture of the Oxford staphylococcus either by the agar cup method or in broth (see Chapter XI) and their relative strengths determined. When two solutions have identical antibacterial activities, the two contain the same number of units of penicillin per unit volume. If a 1:25,000 dilution of the new penicillin gives zones of inhibition in the agar cup method of exactly the same diameter as a 1:1000 dilution of the solution of standard penicillin and if the latter contains 500 International Units per ml., the new solution contains

$$\frac{500 \times 25,000}{1000} = 12,500 \text{ units per ml.}$$

Six other antibiotics require mention. These, with the organisms responsible for their production, are: Streptomycin (*Actinomyces griseus*), Chloromycetin (*Streptomyces venezuelae*), Aureomycin (*Streptomyces aureofaciens*), Gramicidin (*B. brevis*), Tyrocidin (*B. brevis*) and Streptothricin (*Actinomyces lavendulae*). The only one to be synthesised on an economic basis is chloromycetin and so, with it, differentiation of antibiotics from chemotherapeutic substances breaks down.

As streptomycin is ineffective by the oral route and is rapidly excreted, it must be injected intramuscularly, every few hours, or

tion, its concentration in the blood is at a maximum. Penicillin is rapidly excreted from the body, mainly by the urine in which it is found in much higher concentrations than in the blood. Within a few hours of the injection of even a large dose, no penicillin is detectable in the blood. For this reason, solutions of penicillin in water are usually administered either continuously or intermittently at intervals of not more than 4 hours by the intravenous or, more commonly, by the intramuscular route. One way to secure a uniform level of penicillin in the blood without frequent injections is to inject intramuscularly a compound of penicillin and procaine suspended in arachis oil. This acts as a depot from which penicillin is released into the blood over a period of 12 hours or more. The idea that it is essential to administer penicillin in such a way as to secure its presence in the blood and tissues at all times throughout the period of treatment is not now so universally held as in the early days of penicillin therapy. The administration of one or two large doses of penicillin each 24 hours appears to give just as good results as the administration of some six or eight smaller doses in the same period.

If the condition of a patient demands penicillin therapy, the dosage should be adequate. For any condition in which treatment with penicillin is indicated, the dose should not be less than 250,000 units each 24 hours and ten or more times this dose may be required in some cases. The aim should be to secure bactericidal action against the organism responsible for the condition. Lower dosage may be therapeutically useful since bacteriostasis permits the ordinary defence mechanisms to operate, but with prolonged treatment at low concentrations there is a real danger of producing penicillin-resistant strains. A further advantage of high dosage is that, with the resulting high concentration of penicillin in the blood and tissue fluids, there is a better chance of securing an effective concentration of the drug in areas rendered relatively inaccessible to it by the presence of serous membranes, granulation tissue or pus, through all of which penetration takes place with difficulty. Since, even with the highest practicable dose, the concentration in the peritoneal and pleural cavities, in joints, in

the intrathecal space and in abscesses, may still be inadequate, it is often an advantage to supplement general methods of administration by injecting a solution containing say 5000 units per ml. directly into these cavities once every 24 or 48 hours.

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As streptomycin is ineffective by the oral route and is rapidly excreted, it must be injected intramuscularly, every few hours, or

into the body cavities. Chloromycetin and aureomycin are best administered orally. As gramicidin, tyrocidin and streptothricin are toxic, they are suitable only for local application.

Streptomycin is the only substance so far proved capable of killing the tubercle bacillus in the human body. Apart from this, it, like chloromycetin and aureomycin, supplements rather than replaces penicillin as an anti-bacterial agent. The three are of value in combating human infections with bacteria which are either naturally resistant or have acquired resistance to penicillin and the sulphonamides. It will probably become routine practice to precede treatment of a bacterial infection with a test of the organism's sensitivity to sulphonamides and the four antibiotics and to base treatment on the results.

Bacteria exposed to sub-lethal concentrations of streptomycin rapidly develop resistance to it. Treatment of infections other than tuberculosis should, therefore, be with large doses for only a few days. Resistance to chloromycetin and aureomycin is less readily developed.

The main value of chloromycetin and aureomycin lies in their capacity to kill, not only in the embryonated egg and in the infected animal but also in the human body, several varieties of rickettsiæ and viruses. Here chloromycetin is outstanding. It is the treatment of choice in typhus and in the majority, if not in all, the other rickettsial diseases, as well as such virus diseases as psittacosis, atypical pneumonia and lymphogranuloma venereum.

Aureomycin is similar in action to, but less effective than, chloromycetin in rickettsial and viral diseases. It has proved particularly useful in the treatment of lymphogranuloma venereum. One of its disadvantages is its instability in solution.

CHAPTER XI

PRACTICAL METHODS IN CONNECTION WITH ANTIBIOTICS

Penicillin

Testing the Sensitivity of an Organism

We now know, for the majority of the pathogenic bacteria, whether a particular species is sensitive to penicillin or not, but even in the case of such an organism as *Staph pyogenes*, which is classed as sensitive, some strains of much less than normal sensitivity and some which are quite resistant occur. It is, therefore, desirable, if not obligatory, to test the patient's organism against penicillin in every case where penicillin therapy is contemplated.

1. For clinical purposes, a simple and sufficiently accurate method is to spread material such as pus from the patient over a blood-agar plate so as to secure almost confluent growth on one part of the plate and well dispersed colonies on another. Then we divide the medium in the plate into two halves with a sterile knife in such a way as to include on each half both close-set and dispersed colonies. Three drops of a sterile solution of penicillin in saline (5 units/ml) are deposited on one half of the plate and spread over that half with a sterile spreader. The plate is incubated. If the organism is sensitive to such an extent as to render penicillin therapy of value, no colonies will have grown on the half of the plate over which penicillin was spread while a normal growth will be present on the other half. Resistant organisms, if present in the original material, will have grown equally well on both halves of the plate.

2. This, the gutter plate method, is a simple and convenient one for testing and comparing the sensitivity of different bacteria. Two parallel lines, about 1 cm. apart, are cut across the medium

in a plate and the strip so formed is removed. Agar is melted (if the medium in the plate is agar, the strip may be melted) and to it is added sufficient penicillin solution to give a concentration of 10 u. ml. With a sterile pipette, the penicillin-agar so formed is put into the gutter until its surface is level with that of the surrounding medium. A few hours after the agar is set, the plate is



FIG. 41—TEST OF SENSITIVITY TO PENICILLIN BY HALF PLATE METHOD. The plate was uniformly inoculated and then penicillin was spread over the left half.

inoculated in a series of parallel lines drawn at right angles to the gutter and running across it. Each line is composed of a different organism, inoculation being performed with a platinum loop or, more satisfactorily, with a sterile throat swab moistened with a broth culture of the organism to be tested. There is no objection to heavy inoculation when the antibiotic is penicillin. A number of

different organisms can be tested at the same time. After incubation, the relative sensitivity of the various organisms is demonstrated almost diagrammatically. Those which are resistant grow up to and even across the gutter. The growth of those which are slightly sensitive stops short a few millimetres from the edge of the

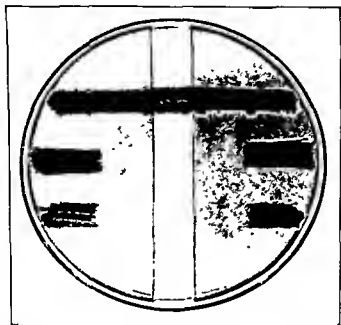


FIG 42 —TEST OF SENSITIVITY TO PENICILLIN BY GUTTER PLATE METHOD
The organism in upper culture is resistant The other two organisms are sensitive

gutter, while very sensitive organisms show no growth within 20 or even 30 mm. of the gutter.

3. This is probably the most accurate method of judging the sensitivity of an organism. In a series of broth tubes each containing a convenient volume of broth (5 ml, for example) halving dilutions of penicillin are made, the range of concentrations commonly being from 4 to $1/64$ u/ml. Each tube is inoculated with one loopful of a broth culture of the organism and incubated

in a plate and the strip so formed is removed. Agar is melted (if the medium in the plate is agar, the strip may be melted) and to it is added sufficient penicillin solution to give a concentration of 10 u/ml. With a sterile pipette, the penicillin-agar so formed is put into the gutter until its surface is level with that of the surrounding medium. A few hours after the agar is set, the plate is

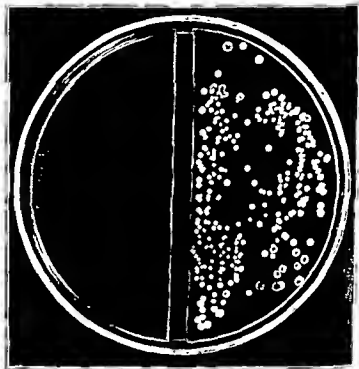


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5-6 mm. in internal diameter) are slightly warmed in a flame and dropped on to the surface of the agar to which they adhere. Accurate dilutions in saline of standard penicillin solution are made so as to secure three dilutions containing 2 u., 1 u., and 0.5 u./ml. respectively. The solution to be tested is diluted so that, as judged by preliminary trial, it contains between 0.5 and 2 u./ml.

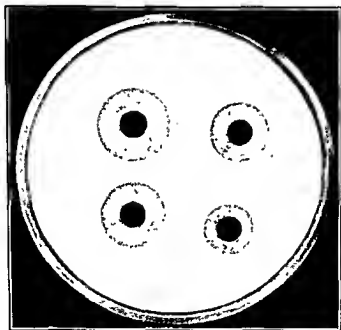


FIG 43—ASSAY OF PENICILLIN BY CUP PLATE METHOD

The four cups are almost filled with the four solutions of penicillin and, after being refrigerated for some hours, the plate is incubated overnight. Penicillin diffuses from the cups into the agar for a distance which varies with the strength of the solution and, where its local concentration is sufficiently high, the bacteria do not grow. The result is that, on the surface of the agar remote from any cup, there is a uniform growth of the organism but there is, around each cup, a circle of agar devoid of growth.

In early tubes in the series, no growth occurs: in later, normal growth. Somewhere between the extremes will usually be found a tube showing slightly cloudy broth, the tube before being clear and the one after quite turbid. We take as our end-point the last tube with completely clear contents.

More accurate results are obtained by a closer series of dilutions. If, for example, 16 ml. of penicillin solution are added to 4 ml. of broth and, after mixing, 16 ml. are transferred to the next tube containing 4 ml. of broth, and so on, the concentration of penicillin in any tube is 20 per cent. less than in the preceding one.

This method is sometimes modified by adding to the broth a fermentable carbohydrate (e.g. glucose) and an indicator such as Andrade's. The end-point is taken as the tube showing a slight pink colour after 24 hours' incubation.

Determining the Strength of a Solution of Penicillin

Since chemical methods are not sufficiently delicate to permit accurate assays of penicillin, biological methods must be used. A unit of penicillin is that amount of penicillin which has the same antibacterial action as a stated weight of a particular sample of standard penicillin. Since there are four different penicillins (I, II, III and IV or F, G, X and K), since commercial products contain these in different proportions and since the activity of each differs *vis-à-vis* different organisms, it is essential to define accurately the methods used to assay a solution. The basis is to compare the antibacterial effect of the solution to be tested and of a solution of known strength against a particular organism, all variables except the penicillin solutions themselves being, as far as possible, excluded. The test organism is almost always a particular strain of *Staphylococcus* known as the Oxford *Staphylococcus*. Only two of the many methods of assay will be described.

1. *Cup Plate Method.*—A few millilitres of a dilution of a broth culture of the Oxford *Staphylococcus* are poured on the surface of an agar plate and then poured off. The plate is incubated for a short time with the lid supported above the bottom to permit the surface to dry. Four glazed porcelain cylinders (9 mm. high and

5–6 mm. in internal diameter) are slightly warmed in a flame and dropped on to the surface of the agar to which they adhere. Accurate dilutions in saline of standard penicillin solution are made so as to secure three dilutions containing 2 u., 1 u., and 0.5 u./ml. respectively. The solution to be tested is diluted so that, as judged by preliminary trial, it contains between 0.5 and 2 u./ml.

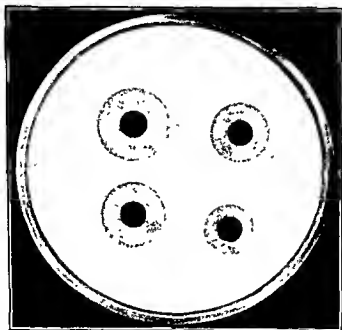


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the other series. From this we calculate the strength of the solution submitted for assay.

Determining the Concentration of Penicillin in Blood and Other Body Fluids

In the early days of penicillin therapy when penicillin was scarce, it was of great importance to determine whether penicillin could be detected in the blood of a patient at the end of the interval between two injections. Since penicillin is now easily and cheaply obtained, we do not purpose to describe any of the methods then used lest it should suggest that the aim in treatment should be to give the least amount which would produce demonstrable effects. We hold strongly that the dose given should be so large that, in a normal person, a high blood concentration will be present during treatment.

The basis of most of the methods employed was to withdraw blood and to test the serum in various dilutions for its power to inhibit the growth of either a *Staphylococcus* or the more sensitive *Strep. pyogenes*.

We still occasionally require to know whether some other body fluid (urine, cerebro-spinal fluid, pleural fluid, pus) contains penicillin and, if so, how much. In such an investigation the range to be covered may be from a fraction of a unit to some hundreds of units per ml. If the fluid is sterile, a series of dilutions of it may be made in broth (1 : 2, 1 : 4, 1 : 8 etc.) and each inoculated with a *Staphylococcus*. From the highest dilution which inhibits the growth of the organism and from the results in a parallel series of dilutions of penicillin of known strength, we can deduce with sufficient accuracy the concentration of penicillin in the fluid.

Another method is to introduce the fluid and suitable dilutions of it into cups or punched holes in a plate spread with staphylococci. The dilution producing a bare zone with a diameter of about 20 mm, provided the zone produced by half this concentration is considerably smaller (about 15 mm), may be taken as containing about 1 u./ml.

A third very simple method is to inoculate the surface of an

The diameter of the circle is proportional (although not directly) to the concentration of penicillin in the cup. The diameters of the bare zones produced by the dilutions of standard penicillin are accurately measured and a graph is constructed, the diameter of the zones (in millimetres) being plotted against the corresponding concentrations of standard penicillin in units/ml. Since the diameter of the zone produced by the solution under test is known, its strength can be read directly from the graph. The diameter of the zone produced by a 1 u./ml. solution is usually 20 ± 5 mm. For really accurate work, various controls must be introduced and many assays made, these being averaged.

A less accurate method of assay does not require any special apparatus such as porcelain cups. The plate is inoculated and dried as before. With a No. 5 cork-borer, sterilised in the flame, four discs of agar are punched out and removed. The cavities left are filled with the four solutions and the plate is incubated. The further operations are exactly as described for the cup-plate method. The results obtained in such an assay are illustrated in Fig. 43. The solutions in the four cups, formed by cutting out discs of agar, were as follows:

2 u./ml.	1 u./ml.
unknown	0.5 u./ml.

The unknown solution was found to contain 1.3 u./ml.

2. *Broth Method.*—A series of dilutions of standard penicillin solution is made in broth and a similar series of dilutions of the solution to be assayed. Every tube is inoculated with 1 loopful of a broth culture of the Oxford *Staphylococcus*. After incubation, a tube which shows faint but definite turbidity in the standard series is selected and an identical one is sought in the other series. If, as usually happens, no exactly matching tube is found, the position of a hypothetical tube which would give an exact match is found by interpolation. Since the solutions of penicillin in these two tubes have identical effects on the organism, their strengths must be identical. We know the concentration of penicillin in the tube from the standard series and hence we know the concentration in the real or hypothetical tube from

Methods Used for Other Antibiotics

Considerable space has been devoted to the methods used in connection with penicillin therapy both because penicillin is still the most valuable antibiotic available at the time this is being written and also because they are special applications of methods capable of wide use.

The other antibiotics to be considered in addition to penicillin are streptomycin, chloromycetin and aureomycin. It is probable that the majority of the methods described in this chapter could easily be adapted to make them suitable for the investigation of these or other similar substances.

Some attention must be devoted to the sulphonamides. The chief points of distinction between them and penicillin are that they are of relatively simple chemical structure and that they are stable. They can be identified and assayed by purely chemical means and there is, therefore, no need to use the indirect methods of assay which penicillin demands.

It is almost as important to determine, in the case of sulphonamides as of penicillin, whether the organism causative of the patient's illness is sensitive or not. Any of the methods described for testing the sensitivity of a bacterium to penicillin can be adapted for sulphonamides but attention must be paid to two points: (1) Most laboratory media contain sulphonamide antagonisers; (2) The inhibition of sensitive organisms by sulphonamides is greatly decreased if the organisms are present in very large numbers. For all tests of sulphonamide sensitivity, the medium (whether broth or agar) should be treated to remove the sulphonamide antagonising substances. This can be accomplished by the method of Harper and Cawston. Horse blood (no other type of blood can be used) is collected in citrate and centrifuged. The plasma is removed and the red cells hæmolyzed by the addition of an equivalent volume of distilled water followed by alternate freezing and thawing. To each litre of medium is added 50 ml. of the hæmolyzed cells and the mixture is incubated for 24 hours. It is then steamed or autoclaved and filtered to remove coagulated protein.

agar plate with staphylococci and to dry as for the cup method. Circular discs of about 15 mm. diameter cut from filter paper and sterilised are saturated with the fluid and dilutions of it and also with a solution of penicillin of known strength (e.g. 10 u./ml.) and dropped into the surface of the agar. The plate is then incubated. Penicillin diffuses from the discs into the agar for distances proportional to the strengths of penicillin used. By finding a dilution of the fluid which gives a similar zone to that produced by the solution of penicillin of known strength, the concentration of penicillin in the fluid may be determined. It should be noted that this method is only applicable to fluids containing concentrations of penicillin higher than about 5 u./ml.

A very simple modification of this method is to deposit a blob of pus on a plate spread with staphylococci. If, after incubation, the blob is surrounded by a clear zone, the pus contained penicillin.

Culture of Fluids Containing Penicillin

If pus from an abscess locally treated with concentrated penicillin is spread on a blood-agar plate, no growth may occur despite the fact that it contains living bacteria. A similar absence of growth may occur in a blood culture performed on a patient receiving large doses of penicillin. The explanation in both cases is that sufficient penicillin is present in the fluid examined to inhibit growth. To demonstrate the presence of living bacteria in such material, we must neutralise the penicillin present. This can most easily be accomplished by adding to the culture medium penicillinase, an enzyme which destroys penicillin. A considerable number of bacteria produce penicillinase. Among these are various coliform bacilli, some strains of *Proteus* and of *Ps. pyocyanea* and some sporing aerobic bacilli. A Seitz filtrate of a broth culture of one of these organisms contains penicillinase and, since it is sterile, it can be added to culture media. Since penicillinase is rather thermolabile it should not be exposed to a temperature exceeding 50° for more than a few minutes.

CHAPTER XII

BACTERIOLOGY OF WATER, MILK AND SHELL-FISH

Water

Water is usually examined in order to determine whether it has been subjected to faecal contamination which, while not dangerous in itself, renders the water liable to become the disseminating agent of various pathogenic bacteria, such as those of the enteric fevers and cholera. The water must be collected in a sterile bottle under conditions which will allow of the examination of a fair and representative sample. It should be examined as fresh as possible. Immediately before being examined the bottle should be vigorously shaken for half a minute.

Counts of colonies resulting from the growth in agar of the bacteria present in water are now rarely made for routine purposes. If they are required, 10 ml. of the water and 10 ml. of a 1 : 10 dilution of it in sterile water, each in duplicate, are added to melted and cooled agar and plates are poured. One pair of plates is incubated at 37° for 2 days and the other pair at 22° for 3 days. The colonies resulting are counted and the result is reported as the number of colonies developed per ml. of water at 22° and at 37°. To avoid a spurious appearance of accuracy, the number should be stated as the nearest number with two significant digits.

The 37° count is of more importance than the 22° count. It is impossible to lay down hard and fast standards, but it may be stated that a water giving rise to more than 50 colonies per ml. after incubation at 37° could rarely be approved and for some types of water as, for example, that from a deep well, a much lower count might give rise to suspicion of contamination.

The majority of the bacteria present in pure water are accustomed to grow at air temperature and either do not grow or grow with difficulty at body temperature. For this reason the

If the gutter or similar method is used to test the sensitivity of an organism to sulphonamide, it is very important not to inoculate the medium heavily. If this is done, even a sensitive organism will grow right up to or possibly on the gutter. The culture used for inoculation should be so diluted as to produce separate colonies rather than a continuous line of growth.

Since sulphonamide diffuses through agar much more slowly than does penicillin, when the gutter or similar technique is used, the plates, after addition of sulphonamide to the gutters, should be kept overnight before inoculation. If this is not done, even light inocula of sensitive organisms may grow up to the gutter, but not on it.

When culturing blood or exudate from patients under sulphonamide treatment to determine whether or not viable bacteria are present, sulphonamide inhibitor in the form of *p*-aminobenzoic acid (*PABA*) must be incorporated in the medium. In view of the wide and often empirical use of sulphonamides in the treatment of undiagnosed fevers, it is desirable to include *p*-aminobenzoic acid as a routine in all broth used for diagnostic blood cultures. Since this substance is heat-stable, it can be added during preparation of the broth and before sterilisation. *P*-aminobenzoic acid neutralises the bacteriostatic effect of many times its equivalent concentration of sulphonamide and 5 mg. per 100 ml. in blood culture broth and 8 mg. per 100 ml. in nutrient agar will be found sufficient for all routine purposes.

✓ CHAPTER XII

BACTERIOLOGY OF WATER, MILK AND SHELL-FISH

Water

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Counts of colonies resulting from the growth in agar of the bacteria present in water are now rarely made for routine purposes. If they are required, 10 ml. of the water and 1.0 ml. of a 1 : 10 dilution of it in sterile water, each in duplicate, are added to melted and cooled agar and plates are poured. One pair of plates is incubated at 37° for 2 days and the other pair at 22° for 3 days. The colonies resulting are counted and the result is reported as the number of colonies developed per ml. of water at 22° and at 37°. To avoid a spurious appearance of accuracy, the number should be stated as the nearest number with two significant digits.

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The majority of the bacteria present in pure water are accustomed to grow at air temperature and either do not grow or grow with difficulty at body temperature. For this reason the

number of colonies which develop at 22° should be very much higher ($\times 10$ or more) than the number developing at 37°. A high 37° count and the approximation of the two counts is very suggestive of contamination.

More important than the total colony count is the estimation of the number of characteristically faecal organisms present. These are known as "indicator" organisms, and the one most usually selected is *Bact. coli*. For the enumeration of this organism we use one bottle (with a tube inverted in it as in a Durham tube), five large and five small Durham tubes. The bottle should contain 50 ml. and the large tubes 10 ml. of double strength lactose bile salt broth. The smaller tubes should contain 10 ml. of ordinary lactose bile salt broth. To the bottle, 50 ml. of the water to be examined are added, to each of the large tubes, 10 ml. and to each of the small tubes, 10 ml. All are incubated at 37° for 48 hours, and are then examined for the production of acid and gas, which is taken as presumptive evidence of the presence of *Bact. coli*.

From the number of tubes showing a positive presumptive *Bact. coli* result in the three volumes examined, it is possible to ascertain, by reference to McCrady's tables (see Chapter XLVIII), the probable number of presumptive *Bact. coli* per 100 ml. of water.

Bact. coli is not the only lactose fermenting organism capable of giving a positive result in lactose bile salt broth and some of the other lactose fermenting bacilli, such as *Bact. aerogenes*, which may be found in faeces but more commonly gains access to water supplies from vegetation or the soil, are of much less sanitary significance than is *Bact. coli*. *Bact. aerogenes* is, however, absent from a pure water and its presence in unusually large numbers is, therefore, suspicious.

Three types of presumptive *Bact. coli* findings should be considered separately—those in which so few are present that the organism may be ignored, whether it be *Bact. coli* or *Bact. aerogenes*, and the water passed; those in which the count is so high that, even if the presumptive result is due to *Bact. aerogenes*, the water should be condemned; and an intermediate type in which the judgment on the water will depend on whether the

organism is, on the one hand, *Bact. coli* or a related, probably faecal, bacillus or, on the other, *Bact. aerogenes* or a related, probably non-faecal, organism. The report of the Ministry of Health fixed the levels at 2 and 10 respectively per 100 ml.—that is to say, a presumptive count of 2 or less should allow a water to pass, while a count of 10 or more should lead to its condemnation. Between 2 and 10 per 100 ml is the border line region. If a presumptive *Bact. coli* count between these levels be obtained it is necessary to isolate the lactose fermenting bacillus and to establish its identity. This is done by plating a loopful from each positive tube or bottle on MacConkey's medium, incubating at 37° for 24 hours and picking colonies for investigation.

Only bacilli which are gelatin liquefying need be *coli*: (1) produces indole; (2) ferment saccharose; (3) gives a positive methyl red test; (4) gives a negative Voges-Proskauer reaction; (5) fails to grow in Koser's medium. If an organism having these characteristics or, at least, Nos. 4, 5 and 6, with any one of the other three, is present to the extent of between 2 and 10 per 100 ml., it should be regarded as *Bact. coli* and the water should be condemned. If the organism responsible for the presumptive positive reaction differs in characteristics 4, 5 and 6, it may be regarded as *Bact. aerogenes*, its presence may be ignored and the water passed. There remain, however, a difficult group of intermediates in which the characteristics are neither those of a typical *Bact. coli* nor of a typical *Bact. aerogenes*. As regards these the bacteriologist must exercise caution, giving a guarded report which will tend to condemn when the presumptive figure is near the upper level (10) and when the characteristics incline towards those of *Bact. coli*, and to approve when the presumptive figure is low and the characteristics approach those of *Bact. aerogenes*.

Since it is generally agreed that the presence in water of *Bact. aerogenes*, except in excessive numbers, is of little hygienic significance, any method which would permit *Bact. coli* but not *Bact. aerogenes* to grow in the tubes of lactose bile salt broth inoculated

number of colonies which develop at 22° should be very much higher ($\times 10$ or more) than the number developing at 37°. A high 37° count and the approximation of the two counts is very suggestive of contamination.

More important than the total colony count is the estimation of the number of characteristically faecal organisms present. These are known as "indicator" organisms, and the one most usually selected is Bact. coli. For the enumeration of this organism we use one bottle (with a tube inverted in it as in a Durham tube), five large and five small Durham tubes. The bottle should contain 50 ml. and the large tubes 10 ml. of double strength lactose bile salt broth. The smaller tubes should contain 10 ml. of ordinary lactose bile salt broth. To the bottle, 50 ml. of the water to be examined are added, to each of the large tubes, 10 ml. and to each of the small tubes, 1.0 ml. All are incubated at 37° for 48 hours, and are then examined for the production of acid and gas, which is taken as presumptive evidence of the presence of Bact. coli.

From the number of tubes showing a positive presumptive Bact. coli result in the three volumes examined, it is possible to ascertain, by reference to McCrady's tables (see Chapter XLVIII), the probable number of presumptive Bact. coli per 100 ml. of water.

Bact. coli is not the only lactose fermenting organism capable of giving a positive result in lactose bile salt broth and some of the other lactose fermenting bacilli, such as Bact. aerogenes, which may be found in faeces but more commonly gains access to water supplies from vegetation or the soil, are of much less sanitary significance than is Bact. coli. Bact. aerogenes is, however, absent from a pure water and its presence in unusually large numbers is, therefore, suspicious.

Three types of presumptive Bact. coli findings should be considered separately—those in which so few are present that the organism may be ignored, whether it be Bact. coli or Bact. aerogenes, and the water passed; those in which the count is so high that, even if the presumptive result is due to Bact. aerogenes, the water should be condemned; and an intermediate type in which the judgment on the water will depend on whether the

may be obtained as to the cleanliness of its method of production and the care with which it has been kept. The most valuable tests are estimations of the number of bacteria growing in agar at 37° and of lactose-fermenting bacilli.

The first estimation is carried out by plating in agar 10 ml. of the milk and the same volume of 1 : 10, 1 : 100, 1 : 1000 and 1 : 10,000 dilutions of the milk in sterile water. If nothing is known of the quality of the milk, it may be necessary to plate the undiluted milk and each dilution up to the 1 : 10,000. For a good milk, the plating of undiluted milk and of the 1 : 10 dilution may be adequate and for a bad milk the plating of the 1 : 1000 and the 1 : 10,000 dilution may suffice. Where there is a legal provision as to the maximum number of bacteria permitted in a milk of designated quality, a single plate may give the required information. If, for example, the maximum permitted is 500,000 per ml. (as in Eire) the plating of the 1 : 1000 dilution suffices as, from it, it is a simple matter to determine whether the milk contains more

incubated at

one showing

the dilution

at 37° from

10 ml. of milk. It is usually stated as the number of bacteria per

In order to estimate the number of lactose-fermenting bacilli, tubes of lactose bile salt broth are inoculated in duplicate with 10 ml. of undiluted milk and of the 1 : 10, 1 : 100 and 1 : 1000 dilution of it in sterile water. The tubes are incubated at 37° for 48 hours.

The production of acid and gas in each of a pair of tubes is taken to signify the presence in the portion examined of lactose fermenting bacilli. The identity or non-identity of these with *Bact. coli* is unimportant, since their source (the faeces of the cow) is similar.

Since it has become an official method of milk examination,

would greatly simplify the bacteriological examination of water. It is now fairly well established that *Bact. coli* (i.e. an organism satisfying criteria 1, 4, 5 and 6 above) can grow at 44°, while *Bact. aerogenes* can not. If we incubate the bottles and tubes of lactose bile salt broth in a water bath at 44°, the development of acid and gas indicates, in the majority of cases, the presence, in the volume of water inoculated, of *Bact. coli* or of closely allied organisms derived from faeces.

Useful as the results of bacteriological examination of a water are, they do not render obsolete its chemical examination nor, above all, do they remove the necessity of a thorough topographical investigation of its source.

The examination of a single sample of water is of little value. A potentially dangerous water may occasionally give very good results. Bacteriological examination is most useful when tests of a given water supply are made repeatedly. Then a bacteriological standard for the particular water may be established and any unusual departure in an upward direction from this is an indication of the access of new contamination.

Members of the enteric group of bacilli have occasionally been isolated from a naturally infected water. Wilson and Blair's medium is most likely to be successful. One method is to mix equal volumes of the water and of the medium, melted, and cooled to 48°, and to pour the mixture into plates. Deep colonies of enteric bacilli in this medium are black. Another method is to inoculate a liquid selective medium, such as tetrathionate broth, with the water and, after incubation, to plate on the surface of Wilson and Blair plates. The cholera vibrio may be found by converting a considerable quantity of the water itself into the medium, by the addition of a concentrated solution of peptone, the further stages being carried out in the manner described in the chapter on Cholera. Very rigorous tests must be applied before a vibrio isolated from water is definitely identified as *V. cholerae*.

Milk

By the bacteriological examination of milk much information

may be obtained as to the cleanliness of its method of production and the care with which it has been kept. The most valuable tests are estimations of the number of bacteria growing in agar at 37° and of lactose-fermenting bacilli.

The first estimation is carried out by plating in agar 1.0 ml. of the milk and the same volume of 1 : 10, 1 : 100, 1 : 1000 and 1 : 10,000 dilutions of the milk in sterile water. If nothing is known of the quality of the milk, it may be necessary to plate the undiluted milk and each dilution up to the 1 : 10,000. For a good milk, the plating of undiluted milk and of the 1 : 10 dilution may be adequate and for a bad milk the plating of the 1 : 1000 and the 1 : 10,000 dilution may suffice. Where there is a legal provision as to the maximum number of bacteria permitted in a milk of designated quality, a single plate may give the required information. If, for example, the maximum permitted is 500,000 per ml. (as in Eire) the plating of the 1 : 1000 dilution suffices as, from it, it is a simple matter to determine whether the milk contains more than 500,000 bacteria per ml. or not. The plates are incubated at 37° for 48 hours and the number of colonies in the one showing nearest to 200 is counted. This number, multiplied by the dilution used, represents the number of colonies developing at 37° from 1.0 ml. of milk. It is usually stated as the number of bacteria per 1.0 ml., but this is inaccurate as not all the bacteria in milk grow under the conditions used and also many of the colonies develop not from one bacterium but from a chain or clump.

In order to estimate the number of lactose-fermenting bacilli, tubes of lactose bile salt broth are inoculated in duplicate with 1.0 ml. of undiluted milk and of the 1 : 10, 1 : 100 and 1 : 1000 dilution of it in sterile water. The tubes are incubated at 37° for 48 hours.

The production of acid and gas in each of a pair of tubes is taken to signify the presence in the portion examined of lactose fermenting bacilli. The identity or non-identity of these with *Bact. coli* is unimportant, since their source (the faeces of the cow) is similar.

Since it has become an official method of milk examination,

both in Great Britain and in Ireland, some account must be given of the methylene blue reduction test, although this is not really a bacteriological test and, in our opinion, can scarcely be considered even scientific.

The basis of the test is that a milk containing a large number of bacteria reduces methylene blue to a colourless compound more quickly than one with few bacteria, but there is not a very close correlation between the number of bacteria present and the rate of decolorisation.

To 10 ml. of milk in a sterile test tube is added 10 ml. of a solution of methylene blue. The tube is closed with a rubber stopper, inverted slowly several times to mix the dye with the milk and kept in the dark in a water bath at 37° – 38° . It is inspected every half hour and the time required to decolorise the blue dye in all but the upper 5 mm. of the column of milk is noted. There are various codes which specify the time during which milks of designated qualities should fail to cause decolorisation. A good quality milk with a low bacterial count may require 7 or more hours; an inferior milk with a high bacterial count may decolorise within 2 hours. Not every sample of methylene blue is satisfactory for this test. Some chemical firms supply tablets of suitable dye from which the solution used in the test can be easily made.

The Prescott and Breed method enables one, within a short time, to form an estimate of the total number of bacteria present in a sample of milk. A slide is laid on a piece of paper ruled in square centimetres. 0.01 ml. of milk is measured with a pipette on to the slide and, with a platinum wire, is spread evenly into a film covering exactly 2 sq. cm. The film is dried in air, treated with xylol to remove the fat and fixed in methyl alcohol. It is stained with an aqueous solution of methylene blue (not Löffler's, in which alkali is present which loosens the film from the slide). By means of a micrometer slide, a suitable ocular and suitable tube extension, the diameter of the field of the microscope is arranged to measure 0.016 cm. which gives a field area of 0.0002 sq. cm. The film is then examined with this combination and the average number of bacteria seen in a number of fields is ascertained.

A clump of bacteria or a chain of streptococci is counted as one since, by the usual plating methods, either would give rise to only one colony. Since the area of the field (0.0002 sq. cm.) is $1/10,000$ of the whole film, and since 0.01 ml. of milk was spread in the film, the number of bacteria per ml. will be (the average number per field) $\times 1,000,000$. This method is rapid, cheap and, on the whole, reliable. Its chief disadvantages are that it does not distinguish living from dead bacteria and that, in good quality milk (with less than 100,000 bacteria per ml.), a number of fields must be examined before any bacteria are found, and hence the results, with such milk, are liable to be inaccurate.

The ordinary dirt bacteria present in milk cause early souring, but are devoid of danger to the human adult, although they

may, however, gain access to the milk either from the milker or other person handling the milk (enteric fever, dysentery, diphtheria and scarlet fever), or from the cow (tuberculosis and undulant fever). The use of Wilson and Blair's medium or tetrathionate broth should render it possible to isolate the enteric bacilli from milk, but success cannot frequently be expected owing to the long incubation period of enteric fever, and hence the considerable interval between the contamination of milk and the

appearance of cases of the disease. Diphtheria bacilli may be

and whether causing scarlet fever or sore throat) by plating on

consumers of milk. *Myc. tuberculosis* is found by centrifuging, at 4,000 revolutions per minute for 20 minutes, 100 to 200 ml of the milk and injecting the deposit into a guinea-pig. After 6 weeks the animal is killed and, if characteristic acid- and alcohol-fast bacilli are found in typical lesions, the tubercle bacillus is

reported to be present. For the detection of *Br. abortus* in milk, 4 ml. of whole milk should be injected subcutaneously into the thigh of a guinea-pig. Some blood should be removed after 3 weeks and again after 6 weeks and the serum tested for agglutinins acting on *Br. abortus*. A titre of 1 : 40 may be taken as establishing the presence of *Br. abortus* in the milk. The animal should be killed in 2 months and cultures attempted from the lymphatic glands draining the area of inoculation and from the spleen. The best media are liver extract agar or agar containing 2 per cent. of glycerol and 5 per cent. of serum. Cultures should be incubated for 7 days in an atmosphere containing 10 per cent. of CO₂.

The results obtained in the bacteriological examination of milk vary enormously. It is impossible under working conditions to obtain sterile cow's milk, but it is quite possible to get milk containing not more than a few hundred bacteria per ml. with no lactose fermenting bacilli in 1 ml. If precautions to prevent access of cow's faeces to the milk are omitted, if improperly cleaned vessels are used, and if the bacteria thus added are encouraged to multiply by keeping the milk for too long a time at too high a temperature (for milk is an excellent culture medium), we need not be surprised to find the total number of bacteria many millions per ml., and lactose-fermenting bacilli present in 0.001 ml., as we may occasionally discover in milk bought in a city in the summer.

One of the most important results achieved by the bacteriological examination of milk is in tracing the influence of each

nd

Milk which has been properly pasteurised should not contain more than 30,000 living bacteria (*i.e.* should not develop more than 30,000 colonies) per ml. and lactose fermenting bacilli should not be present in 10 ml.

Since the majority of pathogenic bacteria likely to occur in milk are killed by pasteurisation (heating to 63°-65° for 30 minutes), it is a matter of considerable importance to be able to

determine whether a milk sold as pasteurised has been adequately heat treated. Phosphatase, an enzyme which is present in fresh raw milk, is destroyed at a slightly higher temperature and slightly more slowly than are the pathogenic bacteria of milk. If phosphatase is found to be absent from a sample of pasteurised milk, it may be assumed that the milk has been adequately heat-treated.

Other fluids may be examined in a manner analogous to that employed for water or milk, the extent of the dilution depending on the probable number of bacteria present. Sewage, for example, contains very much larger numbers of bacteria than does water and therefore the dilutions must be carried much further.

Shellfish

Shellfish are examined to determine whether they are likely to

fore, taken to indicate faecal contamination and so potential danger. The examination should tell not merely whether coliform bacilli are present or not but, if present, their number.

Unfortunately there is no generally accepted method of examination for coliform organisms. The following is an outline of the method we commonly employ. Ten shellfish from each sample are tested. The shells are opened, the shell fluid discarded and the bodies transferred to individual beakers in which they are minced. The volume is made up to a convenient amount, such as 25 ml, with saline. During all these operations precautions are taken, by using sterile equipment and in other ways, to prevent the introduction of contaminating bacteria. Convenient amounts, such as 0.5, 0.1 and 0.02 ml., of the diluted minced body of each shellfish are inoculated in duplicate into large tubes of lactose bile salt broth which are incubated for 24 hours. Tubes with acid and gas after incubation are read as positive. A positive result in both tubes of a pair inoculated with the same volume is taken as signifying the presence of coliform bacilli in that volume. So we can deduce, with a reasonable approximation to the truth, the number

of coliform bacilli in each of the ten shellfish examined. With the figures given above, a positive result in both tubes inoculated with 0.5 ml. indicates the presence of 50 or more coliform bacilli; in both with 0.1 ml., 250 or more coliform bacilli; and in both with 0.02 ml., 1250 or more coliform bacilli per shellfish.

Coliform bacteria other than those likely to be derived from human faeces may be found in shellfish and, since these represent but a very slight potential danger, their presence may be ignored and attention concentrated on presumably faecal types. If suitable dilutions of minced shellfish are inoculated into fluid or liquefied solid media containing bile salt, lactose and an indicator and if the cultures are incubated in a water bath at 44°, the great majority of the lactose fermenting bacteria present will be faecal *Bact. coli*.

There are no universally accepted standards for shellfish but, while it is admitted that no shellfish intended for human consumption, especially in the raw state, should show the presence of as many as 50 coliform bacilli, it is suggested that the following provisional standards may be taken as a reasonable compromise between the ideal and the practical. A batch of 10 shellfish should be considered contaminated to an undesirable extent if more than 7 contain over 50, if more than 3 contain over 250, or if more than 1 contains over 1250 coliform bacilli. Shellfish which have been purified should give very much better results than these.

CHAPTER XIII

BACTERIA IN HEALTH AND DISEASE

Bacteria are either autotrophs (living entirely on inorganic material) or heterotrophs (requiring organic material for their nutrition). The medical bacteriologist is concerned only with the latter and, more particularly, with the limited number of these which are parasitic for man. Among the parasites some species are recognised as pathogenic (disease producing), others as non-pathogenic. The latter, which derive their nourishment from the secretions, excretions and waste products of the body are, despite their close association with their host, essentially saprophytes (forms living on dead organic material). No clear line can be drawn between the pathogenic and saprophytic parasites. The

non-pathogenic bacteria, there are some other species known as facultative pathogens (happily described by Theobald Smith as opportunists). These may exist in or on the body for long periods.

the bacterial flora of the human body.

✓ The skin is constantly receiving bacteria from the air or from objects with which it has come in contact, but the majority of these do not grow on it because of the absence of suitable growth conditions. Few are capable even of surviving on the skin for more than a very short time because of the presence of substance

which are bactericidal for them. Within a few hours of being contaminated with *Bact. coli*, *Salm. typhi* or *Strep. pyogenes*, the skin can free itself completely of these foreign bacteria. Staphylococci and diphtheroid bacilli are much more capable of surviving on the skin and may, in fact, be regarded as constituting the normal skin flora. The staphylococci of the skin are mostly of non-pathogenic varieties. Pathogenic staphylococci usually die quickly on the skin, but the skin of some persons is devoid of the power to kill *Staph. pyogenes* with the result that these persons become carriers of that organism. The conjunctival sac has considerable disinfecting powers so that, unless it is abnormal, few bacteria are to be found in that situation. It is, therefore, wise, before an operation on the eye is performed, to culture a conjunctival swab on blood agar and to postpone operation if *Streptococcus pyogenes*, pneumococci or *Staphylococcus pyogenes* are found; diphtheroid bacilli may be ignored as may also staphylococci of non-pathogenic varieties.

The nostrils serve as a filter for the inspired air, and so the bacteria of this region represent the bacteria of the air and include many varieties of cocci and bacilli together with yeasts and spores of fungi. Healthy carriers of *Staph. pyogenes* usually harbour the organism in the anterior nares. The further we proceed from the exterior, the fewer and more constant are the bacterial species encountered. About the throat, in the presence of no obvious signs of disease, non-hæmolytic streptococci, *N. catarrhalis* and staphylococci very commonly occur, while pneumococci and Friedländer's bacillus are not infrequent. The deeper portions of the respiratory tract, the finer bronchioles and the alveoli of the

complicating
completely

healthy. About their junction with the gums we commonly find non-hæmolytic streptococci, various spirochaetes and lactobacilli.

The alimentary tract presents a highly complicated flora and, a point which can hardly be over-emphasised, one which is comparatively easily altered by changes in diet and other factors. The

contents of the healthy stomach are practically sterile, as are also those of the duodenum, owing to the presence of hydrochloric acid in the gastric secretion, but the intestine contains an enormous number and variety of bacteria. It has been calculated that an adult excretes in the faeces about 3×10^{13} bacteria daily,

Bacilli (chiefly *Cl. perfringens*) are almost always present. The relative proportions of the various organisms are subject to great variations, and it is fallacious to ascribe any ætiological importance to quite wide departures from the normal, although this is frequently done. By the consumption of sour milk, together with excess of lactose, the intestine may be implanted with *L. acidophilus* and the faecal picture transformed from one predominantly Gram negative to one having the majority of the bacteria Gram positive. The closely which are frequently a condition the very become implanted in the intestine as does *L. acidophilus*. The faeces of breast-fed infants contain chiefly Gram positive bacilli, the most important of which are *L. bifidus* and *L. acidophilus*.

tes, various Gram positive
 spirochaetes are found
 urine in the bladder is
 sterile in health. The reaction of the vagina is distinctly acid, and Doderlein's bacillus, an organism probably identical with *L. acidophilus*, is common; *Bact. coli*, faecal streptococci and many other varieties of bacteria can, however, exist there without obvious morbidity. The interior of the uterus and tubes is sterile.

This brief review will serve to indicate the numbers and varieties which as they
 Some of the intestinal bacteria, by synthesising vitamins of group

B, may even be beneficial. When, however, they gain access to unaccustomed tissues, whether as a result of mechanical damage to the protective layer of skin or mucous membrane or of imperfect nutrition, interference with blood supply or other conditions, they behave as pathogens. So *Bact. coli* is a common cause of peritonitis, cystitis and py.
mouth streptococci of
the type of disease pr.

due to the frankly pathogenic organisms, is not, as a rule, infectious. The commensal organisms are, in fact, almost exclusively pathogenic for the host on whom they live, and consequently play an important part as secondary invaders in disease due to other causes whether bacterial (e.g. tuberculosis of the lungs) or metabolic (e.g. diabetes).

The strict pathogens appear to have as their chief function the production of disease in man and animals. When they reach a suitable host they multiply in his body, producing in it their characteristic disease and escape to the body of another victim. We may first consider the methods by which these organisms spread from host to host and the route by which the body is invaded. In temperate climates the majority of epidemic and endemic diseases spread directly or indirectly from individual to individual. The infecting person may himself be suffering from the disease or he may have recovered from it and yet still harbour the causative organism, in which case he is a "carrier". The term carrier is not altogether a satisfactory one since the important thing, in connection with the spread of disease, is not whether a person carries a pathogenic organism somewhere within his body, but whether such an organism leaves his body constantly or intermittently and so becomes capable of infecting others. For this reason the alternative terms "excreter" and "shedder" have been suggested, but neither has yet gained wide acceptance. The carrier condition is always present during convalescence but is not usually of long duration, the organism being eliminated soon after health has been regained. In some cases, however, the organism, normally pathogenic, may become merely parasitic on the particular

others. The carrier state may continue for years and, as a result, many epidemic and endemic diseases—typhoid fever and diphtheria for example—are difficult to stamp out. It occasionally happens that a strict pathogen may be present in the body of an individual for some considerable time before the disease develops. In such cases it is possible that all the conditions necessary for invasion have not yet been fulfilled. Such a person is spoken of as a precocious carrier and is a danger to others as well as to himself. It is also found, during an epidemic, that some persons harbour and excrete the organism for a time without any obvious signs or symptoms of the disease, then or later.

The bacteria or viruses causing many of the commonest infectious diseases, such as diphtheria, scarlet fever, measles and tuberculosis, depart from one host and enter another by the air.

bacteria. A vigorous sneeze may expel as many as 100,000 bacteria-carrying particles. The larger of these soon fall to the ground, but some 4000 of the smaller may remain suspended for more than 30 minutes, travelling during this time for a considerable distance through the air. Before they reach the ground, the water which some of these droplets contain may evaporate leaving bacteria, together with proteins and salts, as droplet-nuclei which may

render transfer in this way particularly likely. Larger drops may fall to the ground and become dry, and the bacteria may lie in the dust, for a longer or shorter time, until stirred up by wind or a misdirected desire for cleanliness which expresses itself by dry sweeping of the floor. Then the bacteria are inhaled with the dust and another victim secured. In hospitals, by the use of masks, we

minimise the risks of transferring infection by droplets and, by oiling floors and bed clothes, we control the spread of bacteria-bearing dust.

were broadcast chiefly by water supplies to which the infected

or indirect contact between the person harbouring the organism

hands which convey the organisms of puerperal sepsis from case to case.

The next group, a very important one, consists of the diseases in which the organism is transferred from individual to individual by the intermediary of a blood-sucking insect. Typhus, spread by the louse, tick or mite, plague by the rat flea and sleeping sickness by the tsetse fly, will serve as examples

Lastly, we have to consider those diseases which occur in the lower animals as well as in man and are commonly spread from the former to the latter. Rabies from the dog, anthrax from the sheep and glanders from the horse are sufficient to demonstrate the importance of this group.

Certain diseases such as diphtheria, typhoid fever and gonorr-

due to invasion of the body by one of several types of influenza virus. Its effects are rendered much more serious by concomitant infection with *H. influenza*, pneumococci and other bacteria. In the comparable disease of pigs, swine influenza, there is a similar association of a virus and a bacterium of the genus *Haemophilus*. The clostridia which have little ability to establish themselves in healthy tissue have the way prepared for them by various types of aerobic bacteria

tylococci and
out gross evi-

degree of pathogenicity and interfere but little with healing. In the absence of satisfactory treatment, these are followed, within a few days, by the chief organisms of sepsis—*Staph. pyogenes* and *Strep. pyogenes*—which, without appropriate treatment, may persist for long periods in the wound. In only a small minority of

ceptible. Each of these factors will now be considered.

bacterium by determining its minimum lethal dose (M.L.D.), which is the smallest number of the bacteria of the smallest volume of a broth culture of them which kills all of a number of similar experimental animals into which that amount is injected. Since the resistance of individual animals varies considerably, a very large number of animals is required to determine the M.L.D. with any degree of accuracy. A more satisfactory way of expressing virulence is as the L D 50, that is the number of bacteria or volume of broth culture required to kill 50 per cent. of the animals.

There is a reciprocal relationship between virulence and dose. When virulence is high, an exceedingly minute dose may cause a fatal infection; when virulence is low, the dose must be much larger.

3. Avenue of Infection

This is occasionally of considerable importance, especially for those organisms of slight aggressiveness. In some cases an organism can only produce its characteristic disease by acting on some definite tissue in the body. The cholera vibrio and dysentery bacilli act almost entirely on the intestinal tract and, when injected subcutaneously, do not produce intestinal disease. The gonococcus can enter the body only by the external genitals or the eye.

4. Defence of Bacteria

Pathogenicity depends, to a considerable extent, on the ability of bacteria possessing it to protect themselves against their host's defensive mechanism. They do so, in some cases, by 'capsules which impede phagocytosis and, in others, by the production of substances which kill leucocytes. Some types of bacteria, faced with the danger represented by their host's antibodies, can produce alternative forms not susceptible to the action of these antibodies. The spirochaetes of relapsing fever offer an excellent example of this. At the time of crisis the serum of the patient is actively destructive to the existing spirochaetes, and yet he may suffer from a series of relapses. In each of these is found a sero-

logically distinct strain of spirochaete produced by the few survivors, and these are not acted upon by antibodies already developed. The gonococcus and tubercle bacillus illustrate another defence of the parasite. They are killed in the animal body by sulphonamides and streptomycin respectively but, if the dose

series of normal untreated animals, they retain this drug-fast

rendering useless these very weapons.

5 Susceptibility of Host

✓ Susceptibility is the reverse of immunity, when one is high, the other is low. The question will be considered in greater detail in the chapters on immunity.

When these five factors, which we have considered, are in favour of the invading bacteria, infection occurs and the bacteria grow in the tissues of the host. It is natural to ask how do they injure the host, how do they cause disease? Bacteria in the body absorb food materials destined for the nourishment of the host's tissues and, by accumulation, act mechanically as foreign bodies. It is not likely that in either of these ways serious damage is inflicted.

on the tissues of their host by bacteria is probably due to their

foreign protein and their products of digestion, the greater part is due to their production of poisonous substances called toxins.

Formerly a sharp distinction was drawn between two classes of toxins—exotoxins and endotoxins. The former were believed to be secretions of the growing bacteria, as they were found in filtrates of broth cultures; the latter were supposed to be contained within the bacterial bodies and to be liberated only by their dissolution. We do not now believe that all exotoxins are secretions for, in some cases (*Cl. tetani* and *C. diphtheriae*), little toxin is found during the phase of active growth, and it may increase markedly while the number of living bacilli is decreasing, and while autolysis is in progress. The two terms can, however, be conveniently retained so long as it is realised that there is no very sharp dividing line between them. In general, exotoxins are active in very minute doses, endotoxins require large doses to produce their effects. The majority of exotoxins exert some

given volume of antitoxic serum can render non-fatal 100 lethal doses of exotoxin, twice the amount of serum will neutralise 200 lethal doses. With endotoxins, however, while a certain amount of antiserum may protect against 5 lethal doses of endotoxin, ten times that amount may fail to neutralise even 10 lethal doses. The number of bacteria known to produce definite exotoxins is small, but newer cultural methods have revealed the production

of typical exotoxins by some organisms, such as staphylococci and streptococci, formerly believed not to produce them.

The poisonous substances produced by bacteria first act locally, giving rise to the primary lesion which in many cases is so

parts of the body where they may affect various tissues. In almost all infections, the brain, cere-

beat and respiration are aff

produced. Degeneration or

in the nervous system, causing paralysis, or in various organs of the body such as the heart, where the muscle cells are affected, and the kidneys, adrenals and liver, where the secreting cells in particular are injured. Further evidences of the general effects of a primary, local lesion are seen in the alterations produced in the circulating blood (anaemia, leucopenia, polymorphonuclear leucocytosis, mononuclear leucocytosis for example).

The type of local lesion varies with the species of bacterium, its virulence, the susceptibility of the host and the particular tissue of the body affected. (The lesion results from the effects of the

host. The effect of the latter is often more obvious to the patient

may be fibrinous, catarrhal, hæmorrhagic, membranous or purulent. The local lesion may remain strictly localised (e.g. a boil) or may be of a spreading character (e.g. cellulitis), the bacteria making their way rapidly through the tissues, aided by the hyaluronidase or other spreading factor produced by them. They may spread to remote parts of the body by means of such natural channels as the ureters or bile ducts, by the lymphatics or by the blood stream. It is probable that the body is invaded by bacteria. When the bacteria (a few per ml) and

✓ bacteremia. When bacteria are constantly present in the blood in large numbers (up to several hundreds per ml.) the term septicemia is used. It is possible that, in septicemia, the bacteria may actually be multiplying in the circulating blood. ✓ Pyemia is the condition resulting from the detachment of portions of a thrombus containing pyogenic bacteria and pus cells. ✓ The portions lodge in remote capillaries where they cause the development of metastatic abscesses. The term oxemia implies that bacterial toxins (without the bacteria themselves) are present in the blood stream.

We have dealt in this chapter with the ways in which bacteria

✓ It will be obvious that there are two chief groups of methods by which the spread of infectious diseases may be checked. ✓ The first is of a general nature and aims at preventing the causal organisms from being conveyed from cases or carriers to fresh individuals (isolation, disinfection, disinfestation, protection of water and food supplies). ✓ The second is personal in character and attempts to increase the individual's powers of resistance by some method of immunisation.

CHAPTER XIV

INTRODUCTION TO IMMUNITY

By immunity we mean the power which the animal body has to resist the action of organic foreign substances, actually or potentially harmful. The term is frequently restricted to resistance against micro-organisms and their products, and it is with this aspect of immunity that we are chiefly concerned.

Immunity is of two main types—that which the individual possesses by virtue of his constitutional and genetic make up (innate or basic) and that which he acquires during the course of his life (acquired).

body of chemical substances called Antigens which do not normally occur there. Antibodies combine specifically with the antigens the presence of which stimulated their production. The antigens with which we are chiefly concerned are those present in

another animal which had been actively immunised. Each of these varieties may be either artificially or naturally acquired.

Innate or Basic Immunity

In the last chapter we saw that, while the majority of bacteria were saprophytes, quite innocuous to living animals, some were strict or facultative pathogens, capable of producing disease once they had entered the body. We must now consider the defences of

the body which tend to keep bacteria from entering the tissues. The chief of these is the integrity of the investing layers of epithelium—skin and mucous membrane. So long as these are intact, they offer an almost complete barrier to the passage of bacteria. It is chiefly as a result of trauma that bacteria effect an entry into the body. The degree of trauma necessary varies with different bacteria; it must be extensive in the case of *Cl. tetani*, but the slightest suffices with *Tr. pallidum*. Next we have the various secretions and excretions which bathe the skin and mucous membranes—sweat, tears, mucus, saliva, gastric and intestinal juices and urine. These act mechanically by washing off bacteria which have adhered to the surface and, in the case of some of the mucous membranes, are aided by directing bacteria away

the most important is p action is assisted by the presence of a substance—Lysozyme—which is capable of killing and dissolving certain bacteria. This substance is found in all the body secretions except urine.

degree of acidity of gastric juice, which almost completely destroys bacteria in the stomach. If the secretion of hydrochloric acid ceases or is diminished, there is a great increase in the number of bacteria found in that organ and also in the duodenum.

There is reason to believe that the effectiveness of these mechanical means of defence may be influenced by diet. It is found that lack of vitamin A increases the liability of experimental animals to bacterial disease, chiefly of the respiratory and alimentary tracts. This is possibly due to alterations in the mucous membranes, whereby they become more permeable and less active in secreting mucus. It has also been observed that guinea-pigs fed on a diet deficient in vitamin C are particularly susceptible to infection by the pneumococcus.

Despite the barriers outlined above, invasion does occur, probably quite commonly, but every invasion does not lead to an infection. Once the skin or mucous membrane has been passed,

the bacteria find themselves exposed to the action of the tissues and tissue fluids which contain lysozyme and a number of other substances injurious to them. These, however, are capable of killing only a minority of the bacteria liable to enter the tissues. The defence of the body against invading bacteria depends mainly on phagocytic cells, that is cells which are capable of ingesting and killing bacteria. Two types of phagocytes are recognised—macrophages and microphages. The former are the cells of connective tissue, whether fixed or free, and the latter are the polymorphonuclear leucocytes. As will be explained later, these cells have very limited powers of ingesting bacteria until the latter have been acted upon by soluble substances called opsonins.

If these dangers have been passed, infection may result provided the bacteria possess sufficient virulence, are present in sufficient numbers and in a suitable tissue, points already considered. Virulence for one animal does not, however, mean virulence for all, and in that lies one of the greatest mysteries of natural immunity. We are really quite ignorant of why syphilis, gonorrhœa, and cholera are exclusively human diseases, or why glanders

action of the toxins of the organisms, as is the case with the rat which is almost unaffected by very large amounts of diphtheria toxin. Even in the same species there are curious inequalities in susceptibility in different races as, for example, the relative immunity of Algerian sheep to anthrax. Some of these, as the extreme severity of measles in aboriginal tribes, are probably due to the lack of inherited immunity. Lastly, it must be recognised that the various tissues of the body are not equally susceptible to

bacterial invasion or to the action of bacterial toxins. Muscles are rarely attacked by the tubercle bacillus; tetanus toxin injures chiefly nerve cells, and the toxin of diphtheria certain epithelial and nerve cells.

Acquired Immunity

Active Immunity Acquired Naturally

It is true of many infectious diseases that a person who has recovered from an attack is incapable of suffering from a second attack for a period which varies with the particular disease from a few months to many years. Such a person, as a result of the attack, has acquired an immunity which he did not previously possess and which suffices to protect him for a longer or shorter time from the bacteria and their injurious products.

It is not essential for the development of immunity that the person should suffer from an actual attack of the disease: sub-clinical infections frequently suffice to cause the development of immunity. Thus, in a community in which diphtheria is endemic, a considerable proportion of individuals who present no history of having suffered from diphtheria are immune. In fact, immunity against diphtheria is probably more frequently due to exposure and sub-clinical infection than to an overt attack of the disease. The proportion of immunes is much greater in higher than in lower age groups. For example, in a particular community, 70 per cent. of individuals at the age of 20 may be found to be immune as compared with 25 per cent. at the age of 2 years. Although there is some evidence suggesting that immunity develops with advancing age as a physiological process, the disparity is due mainly to the fact that, in the higher age group, a greater proportion of individuals have had contact with the diphtheria bacillus, and so have produced antibody to its toxin, than in the lower age group.

Active Immunity Acquired Artificially IMMUNISATION

Immunisation, which may be defined as the artificial stimulation of immunity, is now widely practised and firmly established as a method by which infectious disease may be prevented. The

rationale of all the methods used is the stimulation of the production of antibodies by the introduction into the body of antigens present in micro-organisms, living or dead, or in the toxic products of their metabolism

was inoculated into those desiring protection. The result was the development of smallpox often, but not necessarily, of mild type. Since, at that time, almost everyone suffered from smallpox, it was thought preferable to run the risk at a selected time with a mild form of the disease rather than to wait for chance infection. Although the process of inoculation was artificial, the disease produced was smallpox and the immunity which followed was identical with that following the naturally contracted disease.

The next advance was due to Jenner who discovered that the disease of cattle known as cowpox or vaccinia was transferable to man and that its occurrence gave protection against smallpox. It is probable that the organisms of the two diseases were originally identical and that that of vaccinia has become modified by passage through cattle. The immunity against smallpox afforded by vaccination may, therefore, be regarded as one produced as a result of the action of a living virus modified by passage.

this method must be regarded as impracticable.

This difficulty was overcome by Pasteur who discovered

continued culture in ordinary media at ordinary temperature and culture for shorter periods either in unsuitable media at ordinary

munised person, the latter becomes immune. Since he has not played any part in the development of immunity, his immunity is

immune to a disease some her child. The antibodies in her blood may pass from her circulation to that of the child before its birth by way of the placenta or, after birth, may be transferred by her milk to the infant's digestive tract from which beings the placental

the lower animals the greater portion of this transferred immunity passes by the milk. The antibodies are absorbed from the intestine only for a few days after birth. Later the mucous membrane of the intestine presents

These are examples of animal, either naturally or artificially immune to an organism or a toxin, is injected into a human being, the latter becomes passively immune. This is an

no education ready is injected but they are his body. ly and the

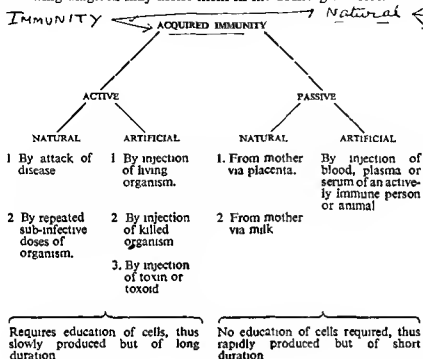
person is no more immune than he was before the serum was injected.

Active immunity is slowly produced but is of long duration. Passive immunity is rapidly produced but is of short duration.

chiefly in the treatment of disease and particularly of acute diseases due to the toxins of bacteria rather than to the bacteria themselves.

Students often find it difficult to distinguish active and passive immunity but they must realise that a true understanding of the

✓ differences in method of production, ✓ rapidity of development and duration of effect is essential as a basis of scientific medicine. The following diagram may assist them in memorising the essentials.



CHAPTER XV

ANTIGENS AND ANTIBODIES

3. The phagocytic cells are probably the most important of the body's defenders but these cells, without the assistance of substances in the plasma, are almost powerless to attack invading bacteria. Plasma, on the other hand, without the help of phagocytes, has some innate powers of destroying bacteria.

When a specific immunity is developed against a particular bacterium, the phagocytes do not increase in numbers or acquire fresh powers, but the plasma undergoes changes by virtue of which its capacity to destroy the bacteria directly, to render them more easily phagocytosed and to neutralise their poisonous properties, is greatly increased.

The science of immunology is largely concerned with the origin and nature of the changes which occur in the plasma of an animal as it becomes immune.

✓ Despite the number and complexity of the substances found in them, the chemical composition of the tissues of the body is wonderfully constant. If any unwanted substance should gain entry to the tissues, efforts are made to remove it as speedily as possible. Simple substances are removed from the site of their introduction by the blood and lymph and are eliminated from the body by the kidneys. More complex substances, and particularly those with large molecules, are removed from the tissues in the same way but they cannot be so easily eliminated from the body. For their disposal a different technique is called into play. This is the elaboration of a substance which can combine chemically or physically with the intruder. If the latter is toxic, the newly developed substance may be regarded as neutralising it and so mitigating its poisonous properties, but even if it is entirely inoffensive a corresponding substance is developed against it.

The foreign substance is called an Antigen and the substance produced against it an Antibody. An antigen might be defined as a chemical substance which, when introduced into the tissues of a living animal, gives rise to the production of an antibody and an antibody as the substance produced by the tissues of a living animal when an antigen is introduced into them. These definitions are not entirely adequate. We can recognise that an antibody has been evoked by a particular antigen only if it interacts in some demonstrable way with that antigen and not with any other antigen. The essential properties of an antigen are, therefore: 1. The ability to stimulate the production of an antibody and 2. The ability to react with that antibody in some demonstrable fashion. These properties may conveniently be referred to as its antigenicity and its reactivity.

ances of high molecular weight into which they are introduced.

The majority of antigens are proteins, but certain other substances, including some polysaccharides and polysaccharide-lipoid complexes, are capable of acting as antigens. Some proteins, including gelatin, are not antigenic, either because of the small size of their molecules or because they lack some essential chemical group.

A considerable number of substances of small molecular weight act as Partial Antigens or Haptens. A hapten is not regarded as a complete antigen because it does not, when introduced into the body alone, stimulate antibody production, but alone it does react specifically with its antibody. Haptens are, therefore, reactive but not antigenic. To convert it into a complete antigen, a hapten must be combined with some other substance, usually a protein, of high molecular weight. The complex stimulates the production of an antibody which reacts with the hapten.

The introduction into the tissues of an animal of intact hæmolytic streptococci results in the formation of antibodies, one of which reacts with hæmolytic streptococci of the same group, but not with those of other groups. If the carbohydrate (C substance) is extracted from streptococci and completely freed of protein, it reacts with the antibody formed as a result of the introduction of

intact streptococci of the same group, but it is incapable alone of stimulating antibody formation. The C substance which is reactive but not, alone, antigenic is a hapten.

Normally, as stated above, a substance native to the tissues of an individual cannot, in them, act as antigen. If, however, the protein of rabbit serum be nitrated, it becomes so altered as to act as an antigen even in the body of the rabbit from which the serum was derived. The antibody produced does not react with rabbit protein, but only with nitrated rabbit protein; the specificity of which resides in the NO_2 group of the molecule.

One of the most important of the characteristics of antigens is their specific relationship with their antibodies. That specificity is based not on the whole antigen molecule but on only a small part of it is suggested by the domination of haptens of low molecular weight over the much larger molecule required to confer antigenicity on them. It would appear that the function of the protein or other substance essential for antigenicity is to carry what is called the determinant group, if this group is not capable of being separated from the rest of the molecule, or hapten if it can be so separated. The specific characteristics of the antibody produced are for the determinant group or hapten and not for the carrier.

Now we must turn to antibodies and, in discussing them, we need not, for the most part, distinguish between those evoked by complete and those evoked by partial antigens.

When an antigenic substance is introduced into the tissues, there is a latent period of several days and then antibody begins to appear in the blood. This increases in amount and reaches a maximum, usually within 3 weeks, after which it diminishes. If, however, a second dose of the antigen is introduced, the antibody concentration in the blood increases to a higher level than formerly. In this way, by successive introductions of antigen, the antibody concentration can be raised to the maximum possible for the animal. When antigen is no longer introduced into the tissues, the amount of antibody in the blood gradually decreases. Ultimately, no antibody may be detected in the serum, but this does not mean that the actively immunised individual has completely

lost his immunity; for years he preserves the ability to produce that antibody more quickly and in larger amount when the specific stimulus is reapplied than can an individual who was never immunised against it.

Unlike insulin or adrenalin, antibody formation is not restricted to any one organ, but is dispersed throughout the body and occurs wherever reticulo-endothelial cells are situated. Among important sites are the lymph nodes from which antibody is carried by the lymph to the blood. Antibody formation is confined to the reticulo-endothelial system because, among the functions of that system, are the synthesis of serum proteins from their constituent amino-acids and the disposal of materials foreign to the body.

It has been known for a considerable time that antibodies are closely associated with the globulin fraction of the blood. We can now go further than this and state that antibodies are globulins, mostly but not entirely γ (gamma) globulin, modified in some way which renders them specific for the antigens which stimulate their production. While it is possible that an already formed molecule of globulin may be converted into a molecule of antibody, it is more probable that it acquires the specific molecular arrangement of an antibody during its formation.

The great majority of antibodies are stable substances which, kept under suitable conditions, remain practically unchanged for years. They are not destroyed at a temperature of 55° , but at higher temperatures, 65° to 70° , they are denatured and lose their power of combining with their antigens.

The importance of specificity in the relationship of antibody to antigen has already been emphasised. At one time it was believed that the basis of this specificity was chemical, so that, when an antigen was brought into contact with its antibody, the two combined forming a new chemical compound. This theory has been replaced by one according to which the combination is physical rather than chemical. It is now believed that the combination is due to the interaction of chains of aminoacids being produced, these electrical charges on their constituent atomic groups are comple-

mentary to those of polar groups on the surface of the antigen molecule. We speak of the "fit" of an antibody to its antigen and such analogies as that of key and lock or mould and cast are often used. These are of value so long as they do not concentrate attention on shape and ignore such questions as that of the distribution of electrical charges which plays an important part.

There is some evidence suggesting that various degrees of correspondence between antigen and antibody may exist. When correspondence is very complete, the union is firm: when it is less complete, the complex is easily broken down into the constituent antigen and antibody.

Despite what has been said about the specificity of antibody and antigen, it is very easy to advance what at first sight appear to be exceptions to this specificity. If we immunise an animal against *Past. pestis*, by injecting it with a suspension of the organism, the serum of the animal will react not only with that organism but also with such diverse bacteria as *Salm. paratyphi B* and pneumococcus of type XIV. The explanation of such examples of apparent lack of specificity is that bacterial and other cells are composed of many chemical substances, each capable of acting as an antigen. The result of the introduction into the tissues of an animal of *Past. pestis* is not the production of an antibody specific for *Past. pestis*, but of a series of antibodies, each specific for one of the antigens of that organism, some of which are shared by the other cells and substances with which, in consequence, the antiserum reacts.

A bacterial cell which is composed of a number of different antigens is sometimes described as constituting an antigenic mosaic. This is not a very good description as it implies that the surface of the bacterium is covered with a very large number of different antigens whereas, although the cell may contain many antigens, only a few of these are present on or close to its surface. If we picture a bacterium as resembling an onion and consisting of successive layers of antigens, we would probably better represent the disposal of its antigens than by regarding it as a mosaic of antigens.

When bacteria are brought into contact with the serum of an

animal immunised against them or, in other words, an antiserum, antibodies in the serum unite with antigens situated at the surface of the cell. Deeply situated antigens are masked by those more superficially placed. ✓ They can react with their antibodies only when the bacteria undergo changes by which antigens formerly deep assume a more superficial position or when the cell is broken up and its antigens go into solution. ✓

The union of antibody and antigen is but the first stage of a process the outcome of which depends largely on the physical state and situation of the antigen and on environmental factors.

✓ If the antigen is in solution, the complex formed is insoluble and so is precipitated. (*precipitation*) Dale's Theory (Page 24)

If the antigen is in its normal position at the surface of a bacterium, it unites with antibody as before. The phenomena which follow depend on a number of circumstances.

✓ If a motile bacillus is used as immunising agent, the union of antibodies with antigens in the flagella causes a thickening of the latter and then, if an electrolyte is present, the flagella of adjacent bacilli adhere together so that the bacteria form clumps, that is, are agglutinated. (*Agglutination*)

✓ If capsulated bacteria are brought into contact with antibodies for the capsular antigens, the first alteration to be observed is swelling of the capsules. This may be followed by agglutination of the bacteria.

✓ Agglutination also follows the union of antibodies with superficially placed antigens of bacteria which are neither flagellated nor capsulated. ✓ But this is not the only result of the action of antibodies on surface antigens. ✓ If polymorphonuclear leucocytes are present, the bacteria become susceptible, as a result of alterations effected by antibodies, to phagocytosis by these cells. This is described as the opsonic effect. ✓ When the bacteria are living and when the antiserum is fresh and consequently rich in an unstable component of serum known as Complement, the united action of antibodies and complement may cause the killing (bactericidal effect) or even the dissolving (bacteriolytic effect) of the bacteria.

It is often convenient to refer to antibodies by names which

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denote the phenomena which follow their union with antigens. So we may speak of precipitin, agglutinin, opsonin, bactericidin and bacteriolysin. Convenient as this usage is, it should not lead us to regard the antibody responsible for one phenomenon as distinct from that responsible for other phenomena. The essential thing is the specific union of antigen and antibody. The obvious features of the reaction, whether these be precipitation, agglutination, phagocytosis, killing or dissolving, are due to secondary effects which are brought about largely by non-specific agencies.

A useful distinction is sometimes made between antibodies which can exert their characteristic effects only if complement is present and those which do not require complement. The former, which include bactericidin and bacteriolysin, are called immune bodies. But again it must be emphasised that the same antibody which may, in the presence of complement, kill the bacteria may, in its absence, merely cause agglutination.

➔ It seems to be universally true that, when antigen and antibody unite, complement, if present, is fixed, even if it takes no part in the subsequent events.

➔ Antibodies are developed not only against the various antigens in the bodies of bacteria, but also against their soluble products such as toxin. The antibody to a toxin, which is called an Antitoxin, unites with toxin and so prevents the latter inflicting damage on susceptible cells. If toxin and antitoxin are present in correct proportions, a precipitation reaction occurs and the insoluble toxin-antitoxin complex is precipitated. ➔ Complement is not required for the neutralisation of toxin by antitoxin but, if present, is fixed.

Antibody formation is a highly important means of defence against bacteria and their products but it is not necessarily the only one. The followers of Besredka believe that certain tissues of the body may become immune to bacteria and their products without the appearance of antibodies in the circulation. While the possibility of tissue immunity cannot be excluded, the probability is that, if it does exist, it is of much less value than that based on the development of antibodies.

CHAPTER XVI

PHAGOCYTOSIS

Of the means by which the body rids itself of bacterial invaders, the most important is based on the activity of certain cells endowed with the power of ingesting these organisms.

Metchnikoff, the discoverer of phagocytosis, described two types of phagocytic cell—microphages and macrophages.

Microphages, better known as polymorphonuclear leucocytes, are normally found in the blood but are able to migrate from it and, after wandering through the tissues, to return to the blood stream. When the body is invaded by bacteria there is usually a marked increase in the number of polymorphonuclear leucocytes in the blood. These cells migrate to the site of invasion and form a protective barrier against further advances of the bacteria. Their bodies, living or dead, constitute the majority of the cells found in pus.

Macrophages are of two types—free and fixed. The former are present free in the tissues and occasionally in the blood, the latter occur in the endothelium of blood vessels, particularly of the liver (Kupffer cells) and bone marrow, and in connective and lymphoid tissues. They play the chief part in the defence of the body against certain bacteria, particularly the tubercle bacillus, but are capable of ingesting many other types of organism and are largely responsible for freeing the blood stream of bacteria which have gained access to it. They also act as scavengers, disposing of dead cells and other waste materials. The fixed macrophages cannot, like the microphages, approach invading bacteria and can therefore ingest only particles which come in contact with them.

The process of phagocytosis by polymorphonuclear leucocytes can be readily studied *in vitro*. If blood cells which have been washed completely free of plasma are incubated with a suspension

of bacteria, phagocytosis *does not* occur. If, however, plasma or serum is added to the mixture of washed cells and bacteria, active phagocytosis takes place on incubation. The presence or absence of phagocytosis is determined by microscopic examination of films prepared from the mixture and stained by Leishman's or other blood stain. If phagocytosis has taken place, many bacteria are present in the cytoplasm of the cells. If there has been no phagocytosis, all the bacteria are situated extra-cellularly.

This experiment suggests that polymorphonuclear leucocytes act as phagocytes of bacteria only in the presence of plasma or serum, but this is not true. If the bacteria are left in contact with

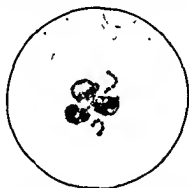


FIG 44.—PHAGOCYTOSIS OF STREPTOCOCCI BY POLYMORPHONUCLEAR LEUCOCYTES

serum for a time and then centrifuged free of it and, after this treatment, are incubated with washed cells, phagocytosis occurs. From this it follows that some substance in the serum acts on bacteria in such a way as to render them capable of being ingested by polymorphonuclear leucocytes. Wright called this substance Opsonin because it made the bacteria palatable for the cells.

Opsonised bacteria attract polymorphonuclear leucocytes to themselves and, when contact has been effected, are ingested by the leucocytes. The first phenomenon, that of attraction, is called positive chemotaxis. When, *in vitro*, bacteria which have not been in contact with serum are used, there is no attraction: there may even be repulsion, that is negative chemotaxis. Cbance encounters

between cells and bacteria must frequently occur but, if the bacteria have not been treated with serum, ingestion does not take place.

Opsonin has many of the properties of an antibody. It can be fixed by bacteria and bacteria so combined with it react in a characteristic way. It differs from most antibodies in two respects: it is more easily destroyed by heat, possibly because the unstable

stances similar in their action to antibodies but innately present in the blood and not resulting from either active or passive immunisation.

Highly pathogenic bacteria, exposed to the action of normal serum and washed cells, probably repel the leucocytes: they certainly do not attract them and are not phagocytosed. If the serum of a human being or animal actively immunised against them is added to the mixture or if the bacteria are treated with such a serum before being added to the cells, phagocytosis takes place. The active ingredient of the serum is called Immune Opsonin or Bacteriotropin. It resembles opsonin in causing bacteria com-

veloped against the surface antigens of the bacteria. It has all the

was extensively used as a method of evaluating the efficacy of vaccine therapy. Two preparations were made. The first contained bacteria, washed white cells and patient's serum, the second the same bacteria and the same washed cells but with normal serum in place of the serum of the patient. After the preparations had been incubated for the same time, films were made and

stained, and the average number of bacteria ingested per polymorphonuclear leucocyte in each was determined. ✓ The opsonic index was obtained by dividing the average in the preparation containing the patient's serum by that in the one containing normal serum. If the index was below 1, the patient's resistance to the organism was regarded as low, if above 1 as high. The significance of the results were not commensurate with the efforts required to achieve them.

When, whether under the influence of opsonins or bacteriotropins, bacteria are phagocytosed, they are exposed to the action of the cell's digestive enzymes. Some types of bacteria are quickly killed and dissolved but others survive for a considerable time. This is shown by the fact that, though phagocytosed, they can still be cultured on laboratory media.

CHAPTER XVII

TOXINS AND ANTITOXINS

While it is probable that all pathogenic bacteria inflict damage on their host by the production of toxic substances, only a limited number have so far been shown to be capable of producing exotoxins in artificial culture. The most important of these are *C. diphtheria*, *Str. pyogenes*, *Staph. pyogenes*, *Shigella dysenteria*, *Cl. botulinum*, *Cl. tetani* and certain other species of the genus *Clostridium*. It is with the toxins of these bacteria and with their antitoxins that we shall deal in this chapter.

Toxins are nitrogenous substances, possibly albumoses, which are soluble and non-crystallisable. They are destroyed by heat, light, exposure to oxygen and various chemical substances. They are present in cultures of the organism grown in broth of suitable composition and can be separated from the bacteria which produced them by filtration through a bacteria-proof filter. Such toxic filtrates are so commonly called toxins that this usage must be accepted, but it should be realised that the true toxin represents a very small fraction of the filtrate.

Certain bacterial toxins are among the most powerful known poisons. One ten thousandth part of a millilitre of a broth culture of *Cl. tetani* suffices to kill a guinea-pig. As small an amount as 2×10^{-8} g. of this toxin in the dry state kills a mouse.

ues which
l cell sub-
It is for
this reason that, when antitoxin is used for treatment, it must, in order to be effective, be administered early in the disease. Once

for the rat and the fatal dose of tetanus toxin per gram of body weight is 200,000 times as great for the hen as for the horse. Tetanus toxin acts mainly on nerve cells and diphtheria toxin on the cells of the adrenals.

Most bacterial toxins are effective only when introduced parenterally. That of *Cl. botulinum* is one of the few which is absorbed from the intestine.

All the known exotoxins are antigenic; their repeated administration in sub-lethal doses stimulates the production of antibodies which are known as antitoxins. The combination of toxin and antitoxin is, like all antigen-antibody reactions, highly specific. Tetanus antitoxin, for example, has no capacity to combine with diphtheria toxin.

When filtrate containing toxin is mixed with serum containing antitoxin, the toxin and antitoxin combine and, if the amount of one is not grossly disproportionate to that of the other, precipitation of a toxin-antitoxin complex occurs. Both the rapidity and the firmness of the union between toxin and antitoxin varies with the sample of antitoxic serum used. The term "avid" was formerly applied to an antitoxin which combined rapidly with toxin, but now it implies that the union with toxin is a firm one rather than

dilution. In one experiment it was found that a rabbit survived an injection of 10 ml. of a particular mixture of toxin and antitoxin but that, when 0.5 ml. of the same mixture was injected into another rabbit, the animal died. In the second case, the dilution of the mixture in the animal's body fluid, which was much greater than in the first case, was sufficient to dissociate the union of toxin and antitoxin so liberating a fatal dose of toxin. It is obvious that an avid antitoxin serum would be more effective therapeutically than a non-avid one but, unfortunately, as we are ignorant of the factors responsible for the avidity of an antitoxin, we are unable to produce avid antitoxic sera when we desire to do so.

toxin-containing filtrate is kept at air temperature for some time, is heated or is acted upon by certain chemical substances, it is found that progressively larger amounts are required to kill

submitting toxin to the action of formalin for some weeks at body temperature. Most of the powerful toxins, including those of *C. diphtheriae* and *Cl. tetani*, can be changed to toxoids.

The discovery of toxoids has been of great importance since it has made possible the active immunisation of human beings against toxins without the dangers inevitably associated with the injection of actual toxin.

Antitoxic sera destined for therapeutic use are usually obtained from horses. A healthy horse receives a series of subcutaneous injections of toxoid of increasing magnitude. This is followed by a further series of injections of toxin. After a few months, when tests have shown that its serum contains a satisfactory amount of antitoxin, the horse is bled. It is not bled to death and, after an interval and a further course of injections, it may be bled again. In this way, with proper care, a horse may continue to yield useful antitoxic serum for a year or more. The blood is allowed to clot and the serum, freed from cells, may be bottled for use after the addition of a small amount of phenol or other antiseptic.

In the early days of antitoxin therapy, crude sera so prepared were used for treatment and, although their potency, measured in terms of units of antitoxin per millilitre, was not great, there are good reasons for believing that their therapeutic effects were greater than those of modern antitoxins, possibly because their

avidity was greater. Partly in order to obtain more units of antitoxin in a given volume and partly in order to avoid the reactions which so frequently follow the injection of crude serum, most antitoxins are now concentrated and refined.

The chief method is based on the fact that antitoxins can be precipitated from solution by ammonium sulphate, freed from this substance by dialysis and redissolved in a volume of saline smaller than that of the serum from which they were obtained. Still further refining is now commonly achieved by partial peptic digestion and heating before precipitation.

Since the concentration of antitoxin present in the sera of different animals immunised against the same toxins differs considerably, it would obviously be unsatisfactory to describe the therapeutic dose of an antitoxic serum in terms of the volume of serum administered. It would also be impracticable to describe the dose in terms of the weight of antitoxin. Some other method of describing the dose is therefore required.

Ehrlich was the first worker seriously to attempt to estimate the potency of an antidiphtheritic serum in terms of its antitoxin content. He first defined a 'unit of toxin', which he called the minimum lethal dose (M.L.D.), as the amount of toxin which, when injected subcutaneously, killed a guinea-pig weighing 250 gm. in 4 days. His unit of antitoxin was that amount which, when mixed with 100 M.L.D. of toxin and injected into a guinea-pig of standard weight, prevented the death of the animal within 4 days.

Ehrlich's methods of standardising toxin and antitoxin were unsatisfactory because of the instability of toxin. As antitoxin is itself much more stable than toxin, antitoxic sera are now standardised by comparing their capacity to neutralise toxin with that of a standard antitoxin. The present unit of diphtheria antitoxin, although based on one of Ehrlich's determinations, is really an arbitrary one. It is the amount of antitoxin contained in a certain weight of a particular antitoxic serum dried and preserved under optimal conditions. All other diphtheria antitoxic sera are standardised directly or indirectly against this serum.

A new antitoxic serum is compared with a standard serum through the intermediary of toxin. Three methods are used in making the comparison.

The first, that of Ramon, is based on the precipitation reaction occurring between toxin and antitoxin. A specimen of a standard antitoxic serum, a specimen of the new serum requiring standardisation and some filtrate containing toxin are required. To a series of tubes, a dilution of standard antitoxin containing exactly 1 unit of antitoxin and decreasing amounts of toxic filtrate are added. Flocculation (or precipitation) first appears in one tube in the series and then spreads to adjacent tubes. The amount of filtrate in the tube in which it first appears is called the Lf dose. Another series of tubes is then prepared. To each is added one Lf dose of the same toxic filtrate and decreasing amounts of the new serum. Since the Lf dose of a toxic broth is the amount which precipitates more rapidly with one unit of antitoxin than any other amount, the tube in the second series in which precipitation first appears contains one unit of antitoxin. This is a rapid and economical method which is now commonly used for preliminary titrations of a new antitoxic serum. It is also used for titrating toxin in a filtrate but, in this connection, it must be realised that toxoid precipitates with antitoxin in exactly the same way as toxin. Two filtrates may have exactly the same Lf value but one may be highly toxic to animals, since the reacting substance is almost entirely toxin, while the other may be almost devoid of poisonous properties because the reacting substance is entirely toxoid. The antitoxic value of a preparation of toxoid is the

The unit of toxin used in the second method of standardisation is the Lr dose. This is the amount of a toxic filtrate which, injected intradermally into a guinea-pig together with one unit of antitoxin, causes a reaction in the form of a red flush 5 mm in diameter within 36 hours. First, using a standard antitoxic serum, the Lr dose of a toxic filtrate is determined.

Then, using the same dose of the same filtrate, the amount of the new serum which permits just the same degree of damage to occur is ascertained: this contains one unit of antitoxin.

The unit of toxin used in the third method of standardisation is the L+ dose of toxin. This is the amount of a toxic filtrate which, when mixed with one unit of antitoxin and injected into a guinea-pig, kills the animal in 4 days. A series of mixtures are made, each containing exactly 1 unit of standard antitoxin and decreasing amounts of toxic filtrate, and each one is injected into a guinea-pig. The amount of the filtrate present in the mixture which caused one of the guinea-pigs to die in 4 days (neither less nor more) is the L+ dose. Another series of mixtures is made, each containing 1 L+ dose of the filtrate and decreasing amounts of the new antitoxic serum, and each one is injected into a guinea-pig. Since, by definition, the L+ dose of a toxin is the amount which, injected together with 1 unit of antitoxin, kills a guinea-pig in 4 days, the mixture injected into the guinea-pig dying in that time must have contained 1 unit of antitoxin.

These three methods are those used for titrating a new diphtheria antitoxic serum. Other methods are used for other sera. For some the mouse may be used as the test animal rather than the guinea-pig and, for standardising the antitoxin to the erythrogenic toxin of *Str. pyogenes*, there is no satisfactory substitute for human skin. An antitoxin to a toxin which is hæmolytic may be standardised by its power to prevent hæmolysis of red blood cells when it is mixed with the toxin and the mixture is added to a suspension of cells.

Despite wide differences in the details of the methods used to standardise different antitoxins, the fundamentals are the same. In every case, one unit of antitoxin is present in that amount of a new serum which, plus a certain volume of a toxic filtrate, produces exactly the same effect as the same amount of filtrate plus one unit of standard antitoxin. The effect may be death of a guinea-pig or mouse within a given period, production of a stated area of erythema in the skin of an animal or man, prevention of lysis of red blood cells or most rapid precipitation.

By international agreement standards for the main antitoxins have been established and specimens of standard sera have been lodged in certain centres throughout the world, so that a manufacturer of such products can accurately assay his products and label them correctly in terms of international units.

CHAPTER XVIII

PRECIPITATION AND AGGLUTINATION

In this chapter it is proposed to deal with the events which follow the union of antibody and bacterial antigen when neither complement nor phagocytic cells are present and, therefore, neither killing nor phagocytosis of bacteria can occur.

Considerable space is devoted to precipitation and agglutination, not because either process appears to be of great practical importance in protecting the body against infection, but because the phenomena, which have been very extensively studied in the laboratory, have served to reveal much of the complex antigenic structure of bacteria and of the nature of antigen-antibody reactions in general.

The essentials of precipitation are easily stated. When the clear serum of an animal immunised against an antigen is mixed with a clear solution of that antigen and when an electrolyte, such as sodium chloride, is present, turbidity develops and, after a time, a precipitate appears and falls to the bottom of the tube. This precipitate, which is composed of the complex formed by the union of antigen and antibody, consists mainly of antibody, that is globulin, with only a very small amount of antigen. It is for this reason that precipitation reactions are of considerable value in detecting, in material examined, the presence of an antigen, even when only very small amounts of it are present, but are much less useful for detecting, in serum, the presence of small amounts of antibody.

It is sometimes convenient, when dealing with precipitation, to use the terms precipitinogen for the antigen and precipitin for the antibody concerned.

In precipitation reactions, the quantitative relationship of anti-

gen and antibody is of great importance. There is an optimum

formation, however, a greater amount of antigen relative to antibody is usually required. If there is a great disparity between the amounts of antigen and antibody present, no precipitation occurs. When the reaction is used for the detection of antigen, a useful method is to layer the solution of antigen on top of undiluted or but slightly diluted serum in a narrow tube as, under these conditions, a precipitate forms as a ring at or close to the junction of the two fluids even when the proportional relationship of antigen and antibody is far removed from the optimum.

The precipitation reaction may be used to detect the presence of bacterial nature in a culture.

the meningococcus can be established by finding that a precipitate forms when the cerebro-spinal fluid is brought in contact with anti-meningococcal serum. The group to which a hæmolytic streptococcus belongs is most conveniently determined by observing with which group antiserum an artificially made extract, containing the C substance of the organism, precipitates.

Apart from bacteriology, the precipitation reaction is extensively employed in medico-legal work as it affords the best method of determining from what species of animal the small amount of blood obtainable from a stain on clothing or weapon was derived.

(2) Agglutination

other electrolytes before they are mixed, no obvious change follows the union. When, however, a trace of electrolyte is added, agglutination takes place.

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In precipitation reactions, the quantitative relationship of anti-

animal agglutinates that organism to a titre of 1 : 5000 and the quite distinct organism, *Salm. typhi*, to a titre of at least 1 : 2500 and frequently of 1 : 10,000 or higher. The explanation of this is

latter.

From what has just been said it follows that the fact that an organism A is agglutinated by the serum of an animal immunised against organism B does not prove that A and B are identical. The identity of the two can be established, but not by direct agglutination. The method used is based on the absorption of agglutinin from an antiserum. If an antiserum is mixed with a dense suspension of the homologous organism, each agglutinin unites with its antigen and, since the latter are attached to the bacteria, when these are removed by centrifuging they take with them their agglutinins. The serum is thus deprived of all its agglutinins. When, instead of the homologous organism, a suspension of a heterologous organism which the serum agglutinates is used, agglutinins acting on it are removed but not necessarily all agglutinins. If the antiserum for an organism A containing antigens 1 and 2 is treated with organism B, the antigens of which are 1, 2 and 3, the serum will not agglutinate either A or B, but if the antiserum for B is treated with A, it will no longer agglutinate A but will still agglutinate B.

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ag:

Test, will be more easily understood from the following table.

If the organism is a motile one and is alive when it is acted upon by agglutinin, the bacteria may be observed to lose their motility before they become agglutinated. Dead bacteria, whether originally motile or non-motile, are just as readily agglutinated as are those which are alive, but it should be noted that the clumps formed by flagellated organisms, living or dead, are larger and looser than those formed by non-flagellated organisms. The former type of agglutination is

Durii patient. For investigating the phenomena of agglutination, the sera of rabbits or other animals, immunised against pure cultures of individual bacterial species, are usually employed. Although agglutination may be observed microscopically, using living bacteria, macroscopic methods, using either living or killed bacteria, are usually preferable. The titre of an agglutinating serum is the highest dilution at which it produces agglutination. It is to some extent affected by the strain of bacteria used, the strength of the suspension and the duration and temperature of incubation. As in the case of precipitation reactions, where special terms are applied to the antigen and antibody concerned, so, in connection with agglutination, the

ing the largest amount of serum may show little or no agglutination. This may be due either to the presence in the serum of some substance, possibly a protein other than globulin, which prevents

which cannot be ignored in agglutination. The existence of these zones of inhibition is a matter of some practical importance, as a serum may fail to agglutinate a bacterium in high concentration i.e. in the earlier tubes in the series, but agglutinates normally in the tubes in which it is more highly diluted.

complete in the case of bacteria of the *Salmonella* genus. A fairly representative species, *Salmonella enteritidis*, contains three groups of antigens—H, O and R. There may be two or three antigens of each group. The H antigens are present in the flagella and, under the influence of their agglutinins, give rise to floccular agglutination. The O antigens, which are somatic, that is associated with the body, give rise, when acted upon by their agglutinins, to granular agglutination. The R antigens are also somatic. O antigens are more superficially placed than R and mask them so that anti-R agglutinins are without effect on the bacteria so long as O antigens are present. H antigens have little or no masking effect on O antigens. Bacteria containing both antigens are usually agglutinated by both anti-H and anti-O agglutinins.

Salmonella enteritidis may produce variants by loss of one or other of the normal groups of antigens. So, a typical strain of *Salmonella enteritidis* may lose its power to produce flagella and, with its loss of flagella, it loses its H antigens. It is no longer agglutinated by anti-H agglutinins but is still agglutinated by anti-O agglutinins. A strain of *Salmonella enteritidis* may lose its O antigens with the result that it is no longer agglutinated by anti-O agglutinins. The loss of O antigens unmasks the R antigens and so this degraded organism is now agglutinable with anti-R agglutinins.

The R antigen is so called because, when O antigen is lost, the colonies of the organism usually appear more or less rough. Such organisms tend to produce suspensions in saline which spontaneously form aggregates similar to those formed when agglutination occurs.

Some species in the genus and particularly *Salmonella typhi* possess an additional somatic antigen called Vi because its presence is associated with virulence of the organism. Vi antigen is super-
 quota of
 anti-O

agglutinins. When it produces a variant through loss of Vi antigen, this variant is agglutinated by anti-O agglutinins but not by anti-Vi agglutinin. The presence of Vi antigen renders *Salmonella typhi* susceptible to the action of certain strains of bacteriophage

Serum.	Antigens against which antibodies are present in the serum	Agglutination of organism*	
		A (containing antigens 1 and 2).	B (containing antigens 1, 2 and 3)
Anti-A	1, 2	+	+
Anti-B	1, 2, 3	+	+
Anti-A absorbed with A	Nil	-	-
Anti-A absorbed with B	Nil	-	-
Anti-B absorbed with A	3	-	+
Anti-B absorbed with B	Nil	-	-

+ = Agglutination

- = No agglutination

Sch. mag. 1910

This experiment shows that organism B possess not only all the antigens of organism A but also at least one additional antigen.

The fact that the serum of a patient agglutinates a particular organism is sometimes taken as proving that the disease from which the patient is suffering is caused by that organism.* While this is often so, the conclusion is not always valid. A person who has once produced a particular antibody is capable of producing it again more quickly and in larger amount than a person who never produced it. A person who suffered from typhoid fever years before may have no demonstrable typhoid agglutinins in his serum. If he receives an injection of a vaccine (a suspension of dead bacteria) of *Salm. typhi*, agglutinins for that organism will

antibody production, which is known as the anamnæstic reaction, should be kept in mind when attempts are made to diagnose a disease from the agglutinating power of a patient's serum.

Our knowledge of the antigenic structure of bacteria is most

strain of *Salm. typhimurium* was again plated and individual colonies were tested by the slide agglutination method against this serum. The serum was found to agglutinate the bacteria in some of the colonies but not those in others. A second rabbit was immunised with the bacteria in one of the colonies which was not agglutinated and its serum was obtained. The same strain of *Salm. typhimurium* was plated out once again and individual colonies were now tested against the two sera. The bacteria in individual colonies were found to be agglutinated either by serum 1 or by serum 2, but not by both. A pure culture of *Salm. typhimurium* has, therefore, produced two types of colony composed of bacteria which can be distinguished serologically from one another.

Next a colony, the bacteria of which were agglutinated by serum 1 but not by serum 2, was plated and the resulting colonies were tested against the two sera. Some (usually the majority) were agglutinated by serum 1 but not by serum 2. The rest were agglutinated by serum 2 but not by serum 1.

In the same way, a colony, the bacteria of which were agglutinated by serum 2 but not by serum 1, was plated. Some of the resulting colonies were agglutinated by serum 2 (usually the majority) and the rest by serum 1.

From these results it may reasonably be assumed that the flagella of one individual bacterium contain antigens of one or other of two types but not both and that, for a few generations,
antigens. Although flagellar
sent in a bacterium, this bac-

to occur in approximately equal numbers.

When other *Salmonella* species were examined in the same way, it was found that, in the case of some, described as diphasic, a similar variation of flagellar antigens occurred, while in others, which were monophasic, only one type of colony could be distinguished.

0 - antigen contains two factors one of which is specific for *S. typhimurium* and the other non-specific.

which lyse organisms containing Vi but not those which have lost it.

A strain of *Salm. typhi* may produce variants in which there is a complete or almost complete loss of one or more of its groups of antigens. In consequence, strains of the organism may be found in which the antigens are:

H, Vi, O, R

Vi, O, R

H, O, R

H, Vi, R

H, R

Vi, R

O, R

R

We now turn to another type of variation, known as diphasic variation, which occurs only in the case of certain *Salmonella* species and which affects only the flagella. This type of variation differs completely from the types already mentioned which were due entirely to loss of antigens and which were irreversible. In this type, it is not a question of loss of antigens but of a replacement of one set of antigens by another set. Further, the variation is reversible, not in the individual bacterium it is true, but in its descendants.

It is impossible, by any method of examination available to us, to determine the nature of the antigens present in the flagella of an individual bacterium but, by examining the mass of bacteria occurring in a well isolated colony and, therefore, presumably descended from a single organism, we can deduce with reasonable certainty the types of antigen present in that organism.

The following account of an experiment is based largely on the methods actually used by Andrewes who discovered this type of variation.

A culture of *Salm. typhimurium* was plated out on agar so as to secure well isolated colonies. After 24 hours' incubation, the bacteria in one colony were used to immunise a rabbit. When the serum of this rabbit became available some weeks later, the same

✓ Characteristics: - 1) Heat stable O or somatic component -

icular species,
The devisers
he number of
phase 1 antigens to exceed twenty-six, but they have already done so with the result that those discovered subsequently to y are now known as z_1, z_2, z_3 and so on.

The H antigens of phase 2, some of which occur in many different species, are normally denoted by Arabic numerals (1, 2, etc.) which allows of unlimited expansion although only a dozen or so have been recognised. Two or three can usually be distinguished in each species. In a few species which are diphasic, the antigens of both phases occur as phase 1 antigens in other species, for which reason small letters are used to denote them.

Any *Salmonella* species may be fully defined by stating its antigenic formula. In this the antigens are given in the following order—O, H of phase 1 and H of phase 2. The formulæ of a number of species, illustrating the points discussed, are given in the table below.

Those gifted with enquiring minds may wonder how it is possible to recognise, in a newly isolated organism found to belong to the genus *Salmonella*, the presence of a particular antigen. Specialists in this type of work must first provide themselves with an antiserum specific for each of the more commonly occurring antigens. Such a serum is produced by immunising a rabbit with

antigens of the organism except the one for which an antibody is required. The unwanted antibodies unite with their antigens in the bodies of the bacteria and, when these are removed by centrifuging, the serum contains only the one antibody and is thus specific for the particular antigen. If, for example, we required a serum containing antibody for antigen 2, we immunise a rabbit with *Salm. paratyphi B* which, as a reference to the table below shows, contains antigens I, IV, V, XII, b, 1 and 2. The serum, therefore, contains antibodies for all these antigens. We treat it

✓ 5. When the sera prepared against the two types of *Salm. typhimurium* were tested against other diphase *Salmonella* species, it was found that serum 1 agglutinated scarcely any colony whereas serum 2 agglutinated a proportion of the colonies produced by several other species. ✓ When the same two sera were tested against monophase *Salmonella* species, it was found, with very few exceptions, that neither sera agglutinated any colony.

The antigens present in the bacteria of colonies of *Salm. typhimurium* which are acted on by antibodies in serum 1 are present in few other species, whereas antigens acted upon by serum 2 are widely distributed throughout various diphase species of the genus *Salmonella*. It was for this reason that the first set of antigens were originally described as specific antigens and the other as group antigens, and bacteria producing one set phase and those producing the other phase. ✓ Further work formerly believed to be peculiar to a species occurred also in some other species and that antigens hitherto believed to be characteristic of the specific phase occurred in both phases and so the phase formerly called specific is now known as phase 1 and the phase formerly called group is now known as phase 2.

We can now state more formally the present state of our

About forty O antigens have so far been distinguished. In some species only one of these antigens is present in easily demonstrable amount, but in others two, three or more such antigens may be recognised. ✓ Identical antigens may be present in several different species so that, if these lose their power of producing another.

etc.).

all letters

s species.

igens are

organisms of which it is composed are virulent. When the O antigens, in the case of the salmonellæ, and the capsules, in the case of the pneumococci, are lost, the organisms become relatively non-virulent and the characteristics of the colonies alter so that these are rough. With other bacteria, such as B. anthracis and Str. pyogenes, the normal colony, consisting of virulent organisms, is rough and the abnormal colony, containing non-virulent organisms, is relatively smooth

✓ Agglutinins can also be produced against other cells, such as the red cells of the blood, by injecting them into an animal of a different species. These are known as hæmagglutinins. In some

known to be present in man and, in various combinations, to be responsible for the four types of human blood.

with dense suspensions of *Salm. abony* and *Salm. paratyphi C*, so absorbing from it antibodies 1, IV, V, XII, b and 1, and leave behind only antibody 2. This treated serum will agglutinate any organism containing, in its flagella, a reasonable amount of antigen 2 and will not agglutinate any organisms the flagella of which do not contain that antigen.

ANTIGENIC STRUCTURE OF CERTAIN SALMONELLÆ

Group.	Species.	Phase variation	Antigenic formula.
A	<i>Salm. paratyphi A</i>	Monophasic	1, II, XII : a
B	<i>Salm. paratyphi B</i>	Diphasic	I, IV, V, XII : b ↔ 1, 2
	<i>Salm. typhimurium</i>	Diphasic	I, IV, V, XII : 1 ↔ 1, 2, 3
	<i>Salm. abony</i>	Diphasic	I, IV, V, XII : b ↔ c, n, x
C	<i>Salm. paratyphi C</i>	Diphasic	VI, VII (Vi) : c ↔ 1, 5
	<i>Salm. oslo</i>	Diphasic	VI, VII : a ↔ c, n, x
	<i>Salm. newport</i>	Diphasic	VI, VII : c, h ↔ 1, 2, 3
	<i>Salm. tennessee</i>	Monophasic	VI, VII : z ₁₁
D	<i>Salm. typhi</i>	Monophasic	IX, XII (Vi) : d
	<i>Salm. enteritidis</i>	Monophasic	IX, XII : g, m

smooth and rough colonies, the cocci in the former having capsules, those in the latter none. The capsule, which contains carbohydrate, confers type specificity and the serum of an animal immunised with capsulated cocci agglutinates only cocci of the same type. The carbohydrate is a hapten. Inoculation of it alone into an animal does not cause the development of antibody. The serum of an animal inoculated with cocci deprived of capsules is species but not type specific: that is, it agglutinates all pneumococci whatever their type.

In the case of *salmonellæ*, pneumococci and a number of other species, the normal type of colony is smooth and the

the serum of all normal animals. It is not a simple substance but

If fresh serum is kept frozen, the amount of complement present falls slowly and useful amounts may still be present after several months. The higher the temperature the more rapid is the rate of disappearance of complement. None may be detectable in serum kept at air temperature after a few days, at body temperature after a few hours or at 55° after a few minutes. Antibodies are very little affected by temperatures up to 55° . In the refrigerator they may show no diminution in amount after many years.

When susceptible bacteria are acted upon by serum containing antibodies and complement, the organisms may not only be killed but may also be partially or completely dissolved. The antibodies

Whether bacteria, after being killed, are dissolved or not depends more on the bacteria themselves than on the antibodies. Some bacteria (*e.g. Bact. coli*) are resistant to the lytic action of antibody and complement and so their bodies, after death, are not dissolved, others (*e.g. vibrios*) are dissolved almost immediately they are killed.

When red blood cells, spermatozoa or cells of the liver or kidney of an animal are injected into an animal of a different species, the latter produces antibodies called cytotoxins which dissolve these cells. The cytotoxins acting on red blood cells, which are known, as hæmolysins, have been the most extensively studied. Hæmolysins also occur naturally, the sera of animals of some species having the power of dissolving the red blood cells of certain other species.

hæmolysis, an apparently clear solution of hæmoglobin.

CHAPTER XIX

THE KILLING AND DISSOLVING OF BACTERIA AND OTHER CELLS

In the last chapter we considered the destruction of bacteria by phagocytic cells and the assistance afforded by substances in the serum to these cells in their fight against bacteria. We now consider the killing of bacteria.

In a serum must contain requisite antibodies are placed somatic antigens of the bacteria. Flagellar and capsular antibodies, acting as they do only on appendages to the bacterial cell, can play no effective part in the killing of bacteria.

The killing of bacteria is often effected by the action of antibodies. There is no objection to this convenient usage provided that it is realised that the killing antibodies are identical with the somatic antibodies demonstrated by other means.

Various theories have been advanced to explain the precise relationship between antibodies and complement in the killing of bacteria. The French school of immunologists, under Bordet, regard an antibody of this class as sensitising its antigen and so rendering it susceptible to the action of complement and, for this reason, they call it Substance Sensibilisatrice. The underlying idea appears to be that antibody so alters the surface of the bacteria that complement is adsorbed. Ehrlich regarded it as a chemical group with valencies for the bacterial antigen on the one hand and for complement on the other and hence he called it Amboceptor. We prefer to use the term Immune Body for an antibody which requires complement for its complete action as this does not imply any particular mode of action.

Complement, called by the French school Alexin, is present in

carried out the following experiment. ^{stage} A suspension of *Past. pestis* was mixed with anti-*Past. pestis* serum and, to the mixture, complement was added. After a period of incubation (during which the bacilli were killed), red blood cells and hæmolytic serum, the complement of which had been destroyed by heat, were added and incubation was again carried out. no hæmolysis resulted. If another organism, say *Salm. typhi*, was substituted for *Past. pestis* or another serum, say anti-*V. cholera*, was used instead of anti-*Past. pestis* serum, hæmolysis occurred. When the

experiment.

This experiment, which has been dignified by the name of the abolished the correctness of the
takes part in the lysis of cells

It was soon realised that the fixation of complement, which
occurs when an antibody combines with its antigen, affords a very

Q = A suspension of bacteria of a different species.

Anti-P = Serum containing antibodies for the antigens of P.

Anti-Q = Serum containing antibodies for the antigens of Q.

C = Fresh normal serum containing complement.

R.B.C. = Washed red blood cells.

H = Serum containing hæmolysin for these cells.

|| = An interval during which the ingredients are kept at 37°.

1. P + Anti-P + C || + R.B.C. + H → No hæmolysis

2. P + Anti-Q + C || + R.B.C. + H → Hæmolysis

3. Q + Anti-P + C || + R.B.C. + H → Hæmolysis

In 1, antibodies in the serum combine with antigens in the bacteria, so fixing complement which is not available to cause lysis of the

have been repeatedly washed in saline are lysed when complement is added. Lysis occurs best at temperatures approximating to that of the body. Complement complex and cannot be

In investigating the and complement, it is advisable to free the cells completely from plasma by repeated washing in normal saline. The hæmolytic serum should be freed from complement by heating to 55° for half an hour. As a source of complement, the fresh serum of a non-immunised animal, usually a guinea-pig, is employed.

The minimum hæmolytic dose (M.H.D.) of a hæmolytic serum is the smallest amount of the serum which, in the presence of cells present in complement, is the the presence of

excess of hæmolysin, causes hæmolysis of all the cells present in a similar is used certain

is required to produce complete hæmolysis. It is usual to titrate hæmolysin in the presence of a great excess of complement and then to titrate complement in the presence of 5 M.H.D. of hæmolysin. In all these titrations, the volume of the suspension of washed red blood cells must be kept constant.

When it was realised that, in order to kill bacteria or to lyse red blood cells, both a specific antibody and another component of serum, complement, was required, a controversy arose as to whether the same complement took part in both reactions.

Ehrlich and Morgenroth maintained that there were several different complements, each adapted to a particular purpose, while Bordet and Gengou held that there was but one complement. The latter workers, in order to establish their contention,

CHAPTER XX

HYPERSENSITIVITY

The term hypersensitivity is applied to a group of conditions in which there is a reaction, either of the whole body or of a particular tissue, to some substance which does not normally give rise to any reaction. These conditions include anaphylaxis, idiosyncrasy or atopy, and allergy but unfortunately there is no general agreement concerning the precise meanings attached to these terms.

We propose to restrict the term anaphylaxis to the condition occurring in experimental animals and, rarely, in man as a result of the parenteral administration of a foreign protein into a body sensitised by a previous introduction of the same substance.

Idiosyncrasy or atopy are the terms we apply to the condition of abnormal sensitivity occurring in man to a variety of substances introduced naturally and not by injection.

Allergy is here confined to hypersensitivity to bacterial products resulting from infection with bacteria or, more rarely, from the artificial introduction of them or their products. This was the original meaning of the term and, in our opinion, is still the correct one, but allergy is now commonly used, especially in popular language, as applicable to almost all forms of hypersensitivity.

Anaphylaxis

As has been seen in the preceding chapters, the tendency in immunity is usually towards the protection of the animal from injurious substances. When we inoculate an animal with a small amount of a bacterial toxin, that animal produces a substance (antitoxin) which will neutralise toxin subsequently injected, and so will protect its cells from injury. In the phenomena which we are now going to examine, the element of protection seems to be absent.

cells sensitised by the hæmolyisin. In 2 and 3, since there is no correspondence between antigens and antibodies, complement is not fixed and so is free to cause lysis of the sensitised cells.

In the reaction, the essential thing is that, when antigen and antibody combine, complement is fixed, even when the two undergo no alteration observable by other means.

The complement fixation reaction has been used for the diagnosis of gonorrhœa, tuberculosis and other diseases, a suspension of the appropriate bacteria being mixed with the serum of the patient. When its most useful practical application, the Wassermann reaction, was devised, *Tr. pallidum* had not been cultured, and so an emulsion of the liver of a syphilitic fœtus, which was rich in spirochætes was employed as antigen. It is found that of such an individual did not. Later, the antigen used was an alcoholic extract of such a liver, diluted with saline, in which the various lipoidal substances present were insoluble and produced a slight turbidity. The test was found to be clinically a reliable one for syphilis and the grounds on which it was based appeared sound. The antigens were supplied by the spirochætes in the liver, the antibodies were present in the patient's serum. This theoretical basis was shattered by finding that an alcoholic extract of normal liver or, better, of normal heart muscle, human or animal, was as efficient an "antigen" as one containing spirochætes. Despite this, the reaction resembles any other antigen-antibody reaction and the resemblance is increased by the finding that, when syphilitic serum is mixed with Wassermann antigen, flocculation occurs.

The reliability of the Wassermann reaction is undoubted and many theories of its rationale have been advanced. The most probable one is that, in syphilis, as a result of the destructive effects of the spirochæte, abnormal lipoids are set free in the body. These act as haptens and, in conjunction with some normal body protein, stimulate the production of antibodies. In the Wassermann and flocculation tests, these antibodies react with the lipoids in the antigen to cause either complement fixation or flocculation.

Sp. B. 1. T. cells - Abnormal lipoids = Haptens

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The fundamental facts of anaphylaxis are easily stated. If a guinea-pig receives an injection of a protein such as horse serum, whether intravenously, intraperitoneally, or subcutaneously and, after a period of from 10 to 14 days, another injection of the same substance, a violent reaction occurs almost immediately, frequently with fatal results. The most important point to be noticed is that the substance

itself, be toxic. A single amount, is in no way injurious to a guinea-pig, but the effects of a second injection are similar to those produced by some violently toxic substance. The animal, as a result of the first injection, after a latent period, becomes sensitive or anaphylactic towards the

for example, will not act as an anaphylactogen. The initial injection is known as the 'sensitising dose'; that which produces the reaction as the reacting, the anaphylactic or the shock dose.

The guinea-pig is the animal most susceptible to anaphylaxis, the rabbit and dog being much less so, while man is, luckily for ourselves, but very slightly susceptible. Much of the experimental work in connection with anaphylaxis has, therefore, been done with guinea-pigs. The dose of foreign protein necessary to sensitise a guinea-pig is very small; one thousandth of a milligram of crystalline egg albumin may suffice. If another injection is made within a week, the animal is unaffected. It does not become anaphylactic for at least 10 days and, if the sensitising dose was large, the necessary latent period before the anaphylactic condition is established is still longer. The reacting dose must be of considerable size—at least one hundred times the sensitising dose when the latter was very small. It may be administered by any route, but it is most rapid in action and a smaller amount suffices if it is given intravenously. The condition of sensitivity, once established, persists a considerable time—in guinea-pigs for 2 years or more. That the reaction is due to the production of an antibody is suggested by the latent period and by the fact that the

✓ injection of a small amount of the serum of an anaphylactic or immune animal into a normal one renders the latter passively anaphylactic. Passive anaphylaxis is also found in the offspring of an anaphylactic mother.

The relation between the anaphylactogen and its antibody is strictly specific, a guinea-pig, sensitised with horse serum, will give no reaction if human serum is subsequently injected. ✓ The exact effect of the reacting dose depends to some extent on its amount and on the route by which it is administered. An acute reaction is generally produced with rapidly fatal results when a sufficient dose is given intravenously, intracerebrally, or intraperitoneally. With the subcutaneous route, the reaction is usually less acute.

An exact description of acute anaphylaxis is difficult, since each species of animal is differently affected. ✓ Every substance causing anaphylaxis will produce exactly the same symptoms in the same species. Horse serum, egg albumin, or vegetable protein cause precisely the same phenomena in guinea-pigs, but the effects of horse serum on a guinea-pig are very different from those of the same substance on a rabbit.

- A guinea-pig, almost immediately after the reacting dose, becomes restless and soon collapses with lowered temperature, urine and faeces being passed. The most marked effects are, however, on respiration, which becomes embarrassed and slowed, and finally ceases with the chest wall immobilised in the position of full inspiration. The heart may continue to beat for some time after the failure of respiration. There are spasmodic twitchings of the limbs, retraction of head and general convulsions. Death, which may occur within 1 minute of the injection, is due chiefly to cessation of respiration caused by extreme contraction of the plain muscle in the walls of the small bronchioles. In rabbits and dogs the outstanding feature is the great fall in blood pressure due, apparently, to abdominal vaso-dilation.

In all animals the appearances are similar to those produced by histamine and consist essentially of contraction of plain muscle and dilatation of capillaries.

✓ The Arthus reaction, which is a slightly different form of

anaphylaxis, occurs in a rabbit submitted to repeated subcutaneous injections in the same situation with a foreign protein such as horse serum. After a few injections have been given, a local reaction, signified by the production of œdema, occurs. The reactions become more and more marked with each succeeding injection and ultimately a firm indurated swelling, in which necrosis may occur, is produced.

✓ De-sensitisation or anti-anaphylaxis may be produced in two ways:—

1. If the animal recovers from acute anaphylaxis, it is insensitive to further injections for some time.

2. Anti-anaphylaxis may also be established by the administration of a second injection within the latent period if this injection be not given within 2 days of the sensitising dose. If repeated injections are given, a refractory state is developed.

✓ The anti-anaphylactic condition is only temporary, and does not last more than 3 weeks in the guinea-pig. ✓ The serum of an anti-anaphylactic animal is capable, occasionally at least, of rendering a normal animal passively anaphylactic.

It is almost certain that the condition is the production of a dose. This is not, however,

an animal may become established immediately. When serum from the anaphylactic animal is injected, a latent interval of some hours is necessary in the case of the guinea-pig, but a shorter time suffices with rabbits and dogs. The simultaneous injection of anaphylactic serum and anaphylactogen is without effect. ✓ Further, the serum is shown not to be the only factor by an experiment in which the blood of an anaphylactic dog was removed and replaced by that of a normal animal. a typical reaction occurred on the injection of the anaphylactogen.

The most probable theory devised to explain anaphylaxis is that of Dale, who regards anaphylaxis as a condition preceding complete immunisation. As the result of the first injection of foreign protein into the blood stream, but are

removed from the blood subsequently and are anchored in or on the surface of tissue cells. ✓ Precipitins contained in the serum of another animal are, after the serum is injected, similarly removed from the circulation. ✓ When the reacting dose is administered a precipitate is formed in or on the cells, and it is this precipitate

to cause symptoms but not death, all the anchored precipitin is used up, and a further injection causes no precipitate. ✓ The

and has the further advantage of bringing it into line with the other facts of immunity.

In many persons the injection of a foreign serum produces a condition known as "serum sickness", in which, after a period of from several days to 2 or 3 weeks, an urticarial eruption appears with some œdema, pyrexia, albuminuria, pains in the joints and glandular enlargement. ✓ These symptoms may occur after a first injection of the serum. Serum sickness is a very common sequel to the administration of therapeutic serum, reactions occurring more frequently and being more marked in individuals receiving a large amount of serum intravenously than when the dose is small or when some other route is employed. ✓ The essential

since it is now known that traces of foreign protein remain in the circulation for a much longer time than was formerly realised. Antibodies may have developed before the foreign protein has

ment of serum sickness.

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It is almost certain that the fundamental factor in anaphylaxis is the production of some antibody as a result of the sensitising dose. This is not, however, the complete explanation, for although an animal may become anaphylactic passively, the state is not established immediately. When serum from the anaphylactic animal is injected, a latent interval of some hours is necessary in the case of the guinea-pig, but a shorter time suffices with rabbits and dogs. The simultaneous injection of anaphylactic serum and anaphylactogen is without effect. ✓ Further, the serum is shown not to be the only factor by an experiment in which the blood of an anaphylactic dog was removed and replaced by that of a normal animal. a typical reaction occurred on the injection of the anaphylactogen.

The most probable theory devised to explain anaphylaxis is that of Dale, who regards anaphylaxis as a condition preceding complete immunisation. ✓ As the result of the first injection of foreign protein, precipitins appear in the blood stream, but are

(Antigen) + (Antibody) = (precipitate)

mucous membranes with excess secretion of tears and nasal mucus

It seems advisable to group these conditions together and to separate them from anaphylaxis. Further work may, however,

sensitive to a number of substances, and in sufferers from hay

examples of hypersensitivity as being special types of anaphylaxis

✓The sensitivity of a person to any suspected substance can be tested either by a small intradermal injection of a solution or extract of the material or by inflicting a scratch on the skin and applying the substance. A positive result is shown by a red flush

increasing doses.

Allergy

The term Allergy which, unfortunately, is used in very different senses by different writers, is here confined to the condition of

the bacteria themselves. Its best examples are seen in the tuberculin and other similar reactions. The mechanism of allergy appears to be quite distinct from that of the local manifestations of anaphylaxis or idiosyncrasy. In the former, the reaction is of an inflammatory nature, and in the latter vasomotor disturbances explain the resulting hyperæmia and œdema.

Occasionally alarming symptoms, which may closely resemble anaphylaxis and which may have a fatal result, very quickly follow a first injection of serum. It seems impossible to explain such phenomena as has been done in the case of serum sickness, since symptoms occur almost immediately and, for the present, they must be considered as examples of idiosyncrasy.

Sensitivity to serum (whether natural or anaphylactic) may be tested by applying a 1 : 10 dilution of the serum to a scratch or by the intradermal injection of 0.2 ml. of a 1 : 100 dilution of the serum. In the sensitive, an urticarial patch or wheal, surrounded by an erythematous area, appears within half an hour. It is stated that desensitisation can be accomplished by injecting 0.1 ml. of a 1 : 100 dilution of the serum subcutaneously and doubling the amount every half-hour, but it is rather doubtful if this procedure would succeed. If serious symptoms develop as a result of any injection of serum, 0.5 ml. of a 1 : 1000 solution of adrenal chloride should be injected subcutaneously without delay as it usually lessens the severity of the attack.

The reaction to serum is not a true allergic reaction and should be borne in mind when considering the treatment by serum in any case requiring it. The rarity of the condition is shown by the fact that, in the 1914-18 War, despite the millions of doses of serum administered, only forty-nine cases of so-called anaphylaxis, with twelve deaths, were recorded among the wounded in British hospitals.

Idiosyncrasy

Idiosyncrasy is a condition in which certain individuals are hypersensitive to certain drugs, or to certain foods, or to certain dusts, or applied to the skin (certain plants such as primula) the reactions may be associated with the respiratory tract giving rise to an asthmatic attack, the gastro-intestinal tract causing vomiting and diarrhoea, the skin producing urticaria or erythema, and the

→ Lactobacteriaceæ	Streptococceæ	Diplococcus Streptococcus
↳ Corynebacteriaceæ	Lactobacilleæ	Lactobacillus Corynebacterium
↳ Achromobacteriaceæ		Listeria
↳ Enterobacteriaceæ	Eschericheæ	Alcaligenes Escherichia Aerobacter Klebsiella
	Proteæ	Proteus
	Salmonelleæ	Salmonella Shigella
↳ Parvobacteriaceæ	Pasteurelleæ	Pasteurella Malleomyces Actinobacillus
	Brucelleæ	Brucella
	Bacterioidæ	Fusobacterium
	Hæmophilææ	Hæmophilus Moraxella Bacterium
↳ Bacteriaceæ		Bacillus
↳ Bacillaceæ		Clostridium
Actinomycetales	Mycobacteriaceæ	Mycobacterium
	Actinomycetaceæ	Actinomycetes
Spirochætales	Treponemataceæ	Borrelia Treponema Leptospira

The outstanding characteristics of the various genera are given below and, as examples, the full name of one species together with the name used in this book when this differs from the one given in the *Manual*.

<u>PSEUDOMONAS</u>	Gram negative, non-sporing rods: usually motile: aerobic produce water-soluble, greenish, fluorescent pigment chiefly found in soil and water some are parasitic
VIBRIO	Gram negative, non-sporing, short, curved rods, occurring singly or united into spirals motile by single polar flagellum aerobic chiefly found in water some are parasitic <i>Vibrio comma</i> (<i>Vibrio cholerae</i>)
<u>SPIRILLUM</u>	Gram negative, non-sporing, long, spiral, rigid organisms motile by tuft of polar flagella aerobic chiefly found in water some are parasitic. <i>Spirillum minus</i>
<u>MICROCOCCUS</u>	Gram positive, non-sporing, non-motile spheres: division occurs in two or three planes aerobic many species produce pigments mostly parasitic. <i>Micrococcus pyogenes</i> (<i>Staphylococcus pyogenes</i>).

CHAPTER XXI

THE CLASSIFICATION OF BACTERIA

In Chapter I, bacteria were divided, on morphological grounds, into five classes—cocci, bacilli, vibrios, spirilla and spirochaetes. Important as morphology is, it is not the only basis of classification and, in the case of bacteria, other methods must be employed to distinguish genera and species. Unfortunately there is little general agreement as to the criteria to be adopted for this purpose. The most widely followed classification is that given in Bergey's Manual of Determinative Bacteriology (Sixth Edition, 1948) a brief outline of which follows.

Bacteria all belong to the Class Schizomycetes. The class is divided into Orders, the orders into Families, the families either into Tribes and these tribes into Genera or directly into genera and the genera into Species. For the identification of a particular organism it is only necessary to give the genus and species. The genus is placed first and its name is written with a capital letter. The name of the species, which is placed second, is written with a small letter.

In the following table we give the chief orders, families, tribes and genera which contain species of medical interest. These represent only a small proportion of the whole, since medical bacteriology is only one branch of the subject. Almost two hundred genera are listed in the Manual.

CLASS Schizomycetes

ORDER	FAMILY.	TRIBE.	GENUS
Eubacteriales	Pseudomonadaceæ	Pseudomonadeæ	Pseudomonas
		Spirillæ	Vibrio
	Micrococcaceæ		Spirillum
			Micrococcus
			Gaffky
	Neisseriaceæ		Sarcina
			Neisseria

<u>KLEBSIELLA.</u>	C	as sole source of carbon: parasitic <i>Klebsiella pneumoniae</i> (<i>Bacterium pneumoniae</i>).
PROTEUS		Gram negative, non-sporing, motile rods: "swarming" growth on moist media: limited fermentative powers with <u>little or no gas</u> : urea decomposed. some liquefy gelatin: some are parasitic. <i>Proteus vulgaris</i>
<u>SALMONELLA</u>		form indole parasitic. <i>Salmonella schottmuelleri</i> (<i>Salmonella paratyphi B</i>)
SHIGELLA		limited do not
PASTEURELLA		Gram negative, non-sporing, non-motile rods: bipolar staining aerobic slight fermentative capacity gelatin not liquefied parasitic. <i>Pasteurella pestis</i>
MALLEOMYCES		Gram negative, non-sporing, non-motile rods with <u>tendency to form filaments</u> and to show branching: parasitic <i>Malleomyces mallei</i>
ACTINOBACILLUS		Gram negative, non-sporing, non-motile rods: <u>pleo-</u> <u>morphic</u> , aerobic in tissues form aggregates resembling granules of actinomycosis parasitic <i>Actinobacillus lignieresii</i>
BRUCELLA		Gram negative, non-sporing, non-motile short rods <u>capsulated</u> . no fermentative capacity parasitic <i>Brucella melitensis</i>
✓ FUSOBACTERIUM		Gram negative, non-sporing, non-motile, long, tapering rods irregular staining <u>anaerobic</u> , parasitic. <i>Fusobacterium plauti-vincenti</i>
HÆMOPHILUS		Gram negative, non-sporing, non-motile rods normally very small but may form threads. require enriched medium parasitic <i>Hæmophilus influenzae</i>
MORAXELLA		Gram negative, non-sporing, non-motile, small rods. aerobic parasitic <i>Moraxella lacunata</i>
<u>BACTERIUM</u>		Gram negative, non-sporing rods.

GAFFKYA.SARCINA.

Gram positive, sporing or non-sporing, non-motile spheres; occur typically in packets of eight; aerobic or microaerophilic; usually produce pigment saprophytic and parasitic.
Sarcina lutea.

NEISSERIA.

Neisseria gonorrhoea.

DIPLOCOCCUS

Gram positive, non-sporing, non-motile spheres or ellipsoids; occur in pairs or short chains some are capsulated, aerobic or anaerobic some require enriched media; ferment carbohydrates; parasitic.
Diplococcus pneumoniae.

STREPTOCOCCUS.

Streptococcus pyogenes.

LACTOBACILLUS

Gram positive, non-sporing, non-motile, long, slender rods microaerophilic; ferment carbohydrates; some parasitic.
Lactobacillus acidophilus

CORYNEBACTERIUM.

Gram positive, non-sporing, non-motile, slender, straight or slightly curved rods; ends frequently pointed or club-shaped, banded or beaded with metachromatic granules; arranged in pairs parallel or at angle; aerobic; parasitic.
Corynebacterium diphtheriae.

LISTERIA

Gram positive, non-sporing, motile, small rods, aerobic, parasitic.
Listeria monocytogenes

ALCALIGENES

Gram negative, non-sporing, motile or non-motile rods non-fermenting. mostly occur in soil or water.
Alcaligenes fecalis.

ESCHERICHIA

Gram negative, non-sporing, motile rods, aerobic, fermenting, producing indole and gas.
 may not act as sole source of carbon: parasitic.
Escherichia coli (*Bacterium coli*)

AEROBACTER

Gram negative, non-sporing, motile, rods, aerobic, fermenting, producing indole and gas.
 carbon, saprophytic or parasitic.
Aerobacter aerogenes (*Bacterium aerogenes*).

The student should note the different meanings of bacterium and bacillus according as these words are written with initial

bacilli.

Several of the more frequently occurring bacteria are known not only by their full scientific names but also by common or vernacular names. As examples of these we may give—the tubercle bacillus, the pneumococcus, the gonococcus and the meningococcus. There can be no objection to this convenient usage which is unlikely to give rise to any doubts as to the precise organism to which reference is made.

<u>BACILLUS.</u>	<u>Gram positive, sporing, motile or non-motile rods:</u> may occur in chains; may be capsulated: in soil and decomposing organic matter. aerobic: some are parasitic. <i>Bacillus anthracis.</i>
<u>CLOSTRIDIUM</u>	
<u>MYCOBACTERIUM.</u>	<u>Gram positive, acid fast, non-sporing, non-motile,</u> straight or slightly curved rods: often stain unevenly aerobic: some grow very slowly: some in soil, water and vegetation: some parasitic. <i>Mycobacterium tuberculosis.</i>
<u>ACTINOMYCES.</u>	<u>Gram positive, non-sporing, non-motile, mycelium</u> r size and
<u>STREPTOMYCES</u>	Mycelium not fragmenting: conidia borne on sporophores: occur in soil. some parasitic. <i>Streptomyces griseus.</i>
<u>BORRELIA</u>	
<u>TREPONEMA</u>	<u>Non-sporing, motile, long, fine, spiral, non-rigid</u> organisms: terminal filament present: stain with difficulty. anaerobic, if capable of cultivation parasitic. <i>Treponema pallidum.</i>
<u>LEPTOSPIRA</u>	<u>Non-sporing, motile, long, fine, spiral, non-rigid</u> organisms: one or both ends bent into hook: stain with difficulty: aerobic: require enriched media in water and parasitic. <i>Leptospira icterohamorrhagiae.</i>

In this edition of the *Handbook*, we employ the majority of the newer generic names given in *Bergey's Manual*. We have not followed Bergey's lead as regards certain organisms and particularly some of those formerly included in the old genus *Bacillus* and now, by him, distinguished by the new generic names *Escherichia*, *Aerobacter* and *Klebsiella*. We have, as a matter of convenience, temporarily included these in the genus *Bacterium* until such time as there is more general agreement as to the various genera to which they should be allocated.

colony. After 24 hours' growth the colour of the colony may be

much more readily than others. For the development of the colour, said to be a lipochrome, oxygen is essential. Pigmentation is usually more intense in cultures grown at 25° than in those incubated at body temperature. A good pigment-producing strain, when kept in culture for some time, may gradually lose its power of producing pigment. On an agar slope, when profusely

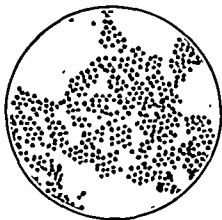


FIG 45.—*Staphylococcus pyogenes* FROM
AGAR CULTURE (X 950)

spread, the staphylococcus gives an abundant growth with a smooth shining surface. On blood-agar, the colonies resemble those on ordinary agar, except that they may be slightly larger. Surrounding each colony is a clear zone of hæmolysis. With some strains the zone may be very wide, with others narrow or absent. Cultures on solid media have a very typical odour.

Staphylococcus pyogenes produces an enzyme, gelatinase, which liquefies gelatin. In a stab culture, liquefaction commences around the stab and progresses until the whole of the medium becomes fluid.

CHAPTER XXII

STAPHYLOCOCCI

Staphylococcus pyogenes (*Micrococcus pyogenes*)

Rosenbach, 1884

This organism is the most important of the genus *Staphylococcus*. The individual cocci are spherical when occurring isolated but, when in groups, may have their adjacent sides somewhat flattened. Their average size is from 0.7 to 0.9 μ ; but exceptional forms, both larger and smaller, may be observed. Careful examination frequently reveals that the larger cocci are those which are about to divide.

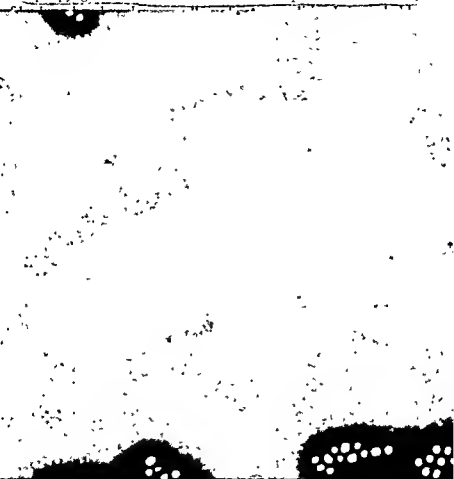
In pus, the cocci occur most typically in irregular masses which have given the name to the group; but single cocci, or those in pairs or short chains of three or four, are frequently seen. In a fresh specimen of pus from an acute lesion many of the leucocytes will be seen to have phagocytosed one or more cocci. The cocci stain well with the basic aniline dyes and retain the stain in Gram's method. *Staphylococcus pyogenes* is non-motile and does not form spores.

This coccus is easy to cultivate on any of the common laboratory media. It grows at temperatures between 12° and 43°, the optimum being about 37°.

On an agar plate the colony has the shape of a segment of a sphere, with smooth shining surface and even outline. Considerable variations in size may be observed, the largest having a diameter of 4 mm. There is little tendency for the colonies to coalesce, unless the plate has been very thickly spread with material, but it may be noticed that, in the more crowded parts of the plate, the colonies are very much smaller than where overcrowding does not occur. The strain of the organism and the type of medium used have also a marked effect on the size of the

and for the leucocidin but not for the enterotoxin or the coagulase may be produced by the immunisation of animals.

Staph. pyogenes is the commonest cause of pustules of the skin,



Staphylococcus aureus, Collargol preparation, Prof Dr A Grumbach, Institute of Hygiene, Zürich

it is possible, by special infections, to cause in animals empirical coccal osteomyelitis and endocarditis essentially similar to the corresponding conditions in man.

Staph. pyogenes spreads directly or indirectly from man to man.

(v) In broth, the *Staphylococcus pyogenes* grows well and causes general turbidity. In a stained preparation of a broth culture, cocci are found singly, in pairs, or in short chains and, but rarely in clusters. After a few days a pellicle forms on the surface of broth and a yellow or brown deposit of cocci collects at bottom of the tube. Broth, free from fermentable carbohydrate

temperature of 90° to kill it. When moist, 10 minutes' exposure at 65° is usually fatal.

Staph. pyogenes produces a number of toxins of which α

man: it is rapidly lethal to experimental animals on intravenous injection. The β toxin is less important in human pathology than is the α toxin: intravenous injection of animals causes death after an interval of days or even weeks. Both these toxins are hæmolytic. Leucocidin, as its name implies, kills leucocytes: it is an important adjunct to the other toxins as it prevents invasion

ice cream and custard. On ingestion of the food, vomiting, with or without diarrhoea, is produced. Coagulase and hyaluronidase, although not, in the strict sense, toxins, may be of importance in the causation of characteristically staphylococcal lesions. The toxins are best prepared by growing the organism in "sloppy" agar (nutrient agar diluted with broth) in an atmosphere containing 10 to 20 per cent. of CO₂. Antitoxins for the α and β toxins

the serum of all normal animals. It is not a simple substance but a complex of at least four different components. Unlike antibody, complement does not increase in amount during immunisation and is very unstable.

If fresh serum is kept frozen, the amount of complement present falls slowly and useful amounts may still be present after several months. The higher the temperature the more rapid is the rate of disappearance of complement. None may be detectable in serum kept at air temperature after a few days, at body temperature after a few hours or at 55° after a few minutes. Antibodies are very little affected by temperatures up to 55° : in the refrigerator they may show no diminution in amount after many years.

When susceptible bacteria are acted upon by serum containing antibodies and complement, the organisms may not only be killed but may also be partially or completely dissolved. The antibodies which, in conjunction with complement, cause dissolution of bacteria are called Bacteriolytins but they are identical with bactericidins and, indeed, with somatic antibodies in general.

Whether bacteria, after being killed, are dissolved or not depends more on the bacteria themselves than on the antibodies. Some bacteria (e.g. *Bact. coli*) are resistant to the lytic action of antibody and complement and so their bodies, after death, are not dissolved; others (e.g. *vibrios*) are dissolved almost immediately they are killed.

When red blood cells, spermatozoa or cells of the liver or kidney of an animal are injected into an animal of a different species, the latter produces antibodies called cytolytins which dissolve these cells. The cytolytins acting on red blood cells, which are known as haemolytins, have been the most extensively studied. Hemo- ^{lytins also occur naturally, the sera of animals of some species having the power of dissolving the red blood cells of certain other species.}

haemolysis, an apparently clear solution of haemoglobin

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s.

f. carbohydrates,

e, mannitol, and

diverse conditions than the majority of non-sporing bacteria. It retains its vitality for months when dried and, in this condition, may require a temperature of 90° to kill it. When moist, 10 minutes' exposure at 65° is usually fatal.

Staph. pyogenes produces a number of toxins of which α and

man: it is rapidly lethal to experimental animals on intravenous injection. The β toxin is less important in human pathology than is the α toxin: intravenous injection of animals causes death after an interval of days or even weeks. Both these toxins are hemolytic. Leucocidin, as its name implies, kills leucocytes: it is an important adjunct to the other toxins as it prevents invading staphylococci from being ingested and killed by polymorphonuclear leucocytes. Enterotoxin, which is responsible for staphylococcal food poisoning, is produced when the organism grows in food rich in carbohydrates and fats such as confectionary, cream, ice cream and custard. On ingestion of the food, vomiting, with or without diarrhoea, is produced. Coagulase and hyaluronidase although not, in the strict sense, toxins, may be of importance in the causation of characteristically staphylococcal lesions. The toxins are best prepared by growing the organism in "sloppy" agar (nutrient agar diluted with broth) in an atmosphere containing 10 to 20 per cent. of CO₂. Antitoxins for the α and β toxins

and for the leucocidin but not for the enterotoxin or the coagulase may be produced by the immunisation of animals.

Staph. pyogenes is the commonest cause of pustules of the skin,



Staphylococcus aureus Collargol preparation, Prof. Dr A. Grumbach, Institute of Hygiene, Zürich

as is possible, by special methods, to make an animal susceptible to coccil osteomyelitis and endocarditis essentially similar to the corresponding conditions in man.

Staph. pyogenes spreads directly or indirectly from man to man.

The source may be a human being suffering from an open lesion—carbuncle, boil or pustule—but is more usually a healthy human carrier. In the carrier condition the organism may be isolated from the anterior nares or, less frequently, from the apparently normal skin. Almost all skin carriers (who may amount to from 10 to 20 per cent. of the population) are also nasal carriers. Not all nasal carriers (30 to 50 per cent. of the population) are skin carriers. The carrier condition, once established, may be of very long duration.

When a *Staphylococcus* has been isolated from a suspected carrier or from a lesion with mixed bacterial flora, it may be a matter of great importance to determine whether it is a *Staph. pyogenes*, that is a staphylococcus pathogenic for human beings. A pathogenic *Staphylococcus* is usually well pigmented, hæmolytic, mannitol-fermenting and gelatin-liquefying, but by far the best method of determining the pathogenicity of a strain is to apply the coagulase test. Almost without exception, coagulase positive staphylococci are pathogenic and pathogenic staphylococci are coagulase positive. The test is best applied by adding to 0.5 ml. of a 1 : 10 dilution in saline of citrated or oxalated human plasma, 5 drops of a broth (not glucose broth) culture of the organism and incubating for 2 to 3 hours. A coagulase positive *Staphylococcus* so tested causes the plasma to coagulate provided a suitable sample of plasma has been employed. Only by testing with a known coagulase positive *Staphylococcus* can a suitable plasma be selected. The results are little inferior if a colony of the organism to be tested is suspended in the dilute plasma.

Although it is possible by serological means to distinguish three or more distinct types of *Staph. pyogenes*, this method of typing is scarcely adequate to assist in tracking the source of infection to a previous case or carrier. Phage typing by methods similar to those used in the typing of *Salm. typhi* are much more promising, over 20 types having so far been distinguished. While all the technical difficulties have not yet been overcome, there is little doubt that the method will be of increasing value in the future.

Much of the reputation of vaccine therapy was based on the undoubted success of staphylococcal vaccines in the treatment of boils and other staphylococcal lesions and, even more, in the prevention of their recurrence. A later development was the employment of staphylococcus toxoid, prepared by treating a toxic filtrate with formalin. Treatment of acute infections with antitoxic sera, while occasionally of value, was never as successful as might have been expected.

Sulphanilamide has very little action on *Staph. pyogenes* but some of the newer sulphonamides are undoubtedly of value in the treatment of staphylococcal infections. Owing to the almost spectacular success of penicillin in combating *Staph. pyogenes* in the body, there should be no hesitation in employing this antibiotic in preference to sulphonamides. Since some 10 per cent. of strains are relatively or completely resistant to penicillin, the infecting strain should be tested for sensitivity prior to treatment. For the treatment of infections with penicillin-resistant strains, streptomycin, chloromycetin or aureomycin may be used.

Other Staphylococci

Many staphylococci, other than the coagulase positive *Staph. pyogenes*, may be found about the human body, particularly in the nose and on the skin. These include *Staph. albus*, *Staph. citreus* and *Staph. epidermidis*. These are all coagulase negative and, for the most part, fail to ferment mannitol or to liquefy gelatin. On agar, the colonies of *Staph. albus* are white and those of *Staph. citreus* lemon yellow. *Staph. epidermidis* grows more slowly than the other staphylococci and produces smaller colonies. These organisms are not completely devoid of pathogenicity although they are much less actively pathogenic than *Staph. pyogenes*. They may be responsible for complications in lesions caused by other bacteria, for delay in the healing of wounds and for such minor inconveniences as stitch abscesses.

Gaffkya tetragena, a Gram positive coccus, frequently capsulated and occurring most characteristically in regular groups of four (tetrads), despite its differences from staphylococci, may be

considered with these organisms. *G. tetragena* is definitely although not very actively, pathogenic. It is occasionally responsible for meningitis, empyema and other types of inflammation and has even been isolated from the blood stream. It is, however, more commonly a secondary invader of tissues damaged by more active pathogens. It is not infrequently found, for example, in the sputum in phthisis. (1.3).

CHAPTER XXIII

STREPTOCOCCI

Hæmolytic. β

Non-hæmolytic.

Cocci which divide in only one plane, producing chains of adherent organisms, are called streptococci. It should be realised that the term is purely a morphological one and that the species of this large genus have different cultural and biochemical characteristics and differ widely in their natural habitats and pathogenic properties.

A very important method of classifying streptococci is accord-

defined zone of hæmolysis: the β streptococci give rise to wide zones of clear hæmolysis around their colonies: the γ streptococci do not cause any alteration in the medium. Very commonly, the slight hæmolysis produced by α streptococci is ignored and streptococci are described as hæmolytic (β) or non-hæmolytic (α and γ). The term *Streptococcus viridans* is often applied to α streptococci but, as this name is not valid for any species, it is preferable to refer to these organisms as viridans streptococci.

Classification by fermentative capacity was used, not very successfully, for a time, but has now been almost completely replaced by surer serological methods.

Serological methods of classifying streptococci have been applied chiefly to hæmolytic streptococci and among these the strains which are pathogenic for human beings have been most intensively studied.

Lancefield discovered that it was possible to extract from streptococci a polysaccharide hapten. If the clear serum of an animal immunised against a particular strain of streptococcus is

mixed with a clear solution of the polysaccharide extracted from streptococci of the same strain, a precipitate forms. All other streptococci, the extracts from which are precipitated by this serum, are assigned to the same group. In this way the hæmolytic streptococci may be divided into twelve groups—A, B, C, D etc. All the organisms in a particular group contain an antigenically identical polysaccharide which precipitates with the same serum. Some non-hæmolytic streptococci contain the same polysaccharide as the one accepted as characteristic of a particular group of hæmolytic streptococci. For this reason they are included in the same group. This explains why both hæmolytic and non-hæmolytic streptococci may occur in a single group as, for example, in group D.

A method of serological classification which is based on agglutination was introduced by Griffith. The marked tendency of most streptococci to undergo spontaneous agglutination renders agglutination techniques, as applied to other bacteria, unreliable in the case of streptococci. Griffith overcame most of the difficulties by using the slide agglutination method. In this way he and his followers were able to distinguish 30 serologically different types of hæmolytic streptococci isolated from human lesions. These are known as types, 1, 2, 3, 4 etc. Since streptococci of Lancefield's group A (*Str. pyogenes*) are by far the commonest streptococci pathogenic for man, it is not surprising to find that 26 of Griffith's types belong to group A as compared with 3 belonging to group C and 1 to group G. Various antigenic substances are responsible for the type specificity of streptococci. These include proteins, polysaccharides and substances of undetermined composition. Typing should be carried out on freshly isolated and fully virulent strains as some of the substances on which type specificity depends may be absent from old, avirulent strains.

Further investigation will undoubtedly show that the streptococci of groups other than A may similarly be divided into a large number of types.

In diagnostic bacteriology it is usually quite sufficient to deter-

mine whether a strain of *Streptococcus* under investigation is hæmolytic or not and, if hæmolytic, the group to which it belongs. The chief value of typing is to assist in tracing a *Streptococcus* found responsible for an infection to its source in an earlier case or in a carrier.

Streptococcus pyogenes

Rosenbach, 1884

order 20 bacillales
F 3 at H. H. H. H.
7 12 3 12 12 12
12 12 12 12 12 12
12 12 12 12 12 12
12 12 12 12 12 12

The specific name *Streptococcus pyogenes* is now given to any hæmolytic streptococcus which belongs to Lancefield's group A. This organism is by far the most important of the hæmolytic streptococci found in human disease. The individual cocci vary from 0.5 to 1.0 μ in diameter, and larger cocci, which are especially common in old cultures, are to be regarded as abnormal and involution forms. In shape they are spherical, but frequently adjacent cocci in a chain are slightly flattened. They are non-motile and do not form spores. They stain well with the ordinary aniline dyes and are Gram positive. Capsules are not usually found in films of *Str. pyogenes* prepared from ordinary cultures, but it is possible to demonstrate the presence of capsules in fully virulent strains, provided they are grown in serum broth and the examination is made after only 2 or 3 hours' incubation.

When a stained film of streptococcal pus is examined, the cocci are found to be present, some in pairs and some in short or long chains, each consisting of up to 20 or 30 cocci. In early lesions the diplococcal form predominates, but in pus from abscesses, long chains are common.

Str. pyogenes grows on most of the ordinary laboratory media, but never so well or so luxuriantly as the staphylococci. Occasionally it is impossible to grow it in primary culture on media which have not been enriched by the addition of blood, serum or ascitic fluid. Later cultures, however, grow without these but in any medium, serum or ascitic fluid and 0.5 per cent of glucose promote good growth. The fact that *Str. pyogenes* grows on blood-agar containing a 1 : 500,000 concentration of gentian violet on which staphylococci, aerobic sporing bacilli, diphtheroid bacilli,

mixed with a clear solution of the polysaccharide extracted from streptococci of the same strain, a precipitate forms. All other streptococci, the extracts from which are precipitated by this serum, are assigned to the same group. In this way the hæmolytic streptococci may be divided into twelve groups—A, B, C, D etc. All the organisms in a particular group contain an antigenically identical polysaccharide which precipitates with the same serum. ✓ Some non-hæmolytic streptococci contain the same polysaccharide as the one accepted as characteristic of a particular group of hæmolytic streptococci. For this reason they are included in the same group. This explains why both hæmolytic and non-hæmolytic streptococci may occur in a single group as, for example, in group D.

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Streptococcus pyogenes

Rosenbach, 1884

order to be made clear
F. & J. W. H. & C. Co.
T. & J. W. H. & C. Co.
L. & J. W. H. & C. Co.
W. & J. W. H. & C. Co.

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certain other bacteria and yeasts do not grow, facilitates its isolation from situations in which large numbers of such organisms are present. ✓The presence of bile in the medium inhibits the growth of the organism. ✓*Str. pyogenes* is aerobic but, like the majority of streptococci, grows better under anaerobic conditions. It grows best between 35° and 38° , and no growth occurs below 18° .
 (c) On agar the colonies are visible in 24 hours. ✓They are circular in shape, raised in the centre, opaque and greyish in colour and, when magnified, are seen to be finely granular. Their size rarely exceeds 1 mm. in diameter. The colonies show very little tendency

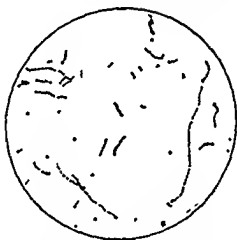


FIG. 46—*Streptococcus pyogenes* FROM BROTH CULTURE ($\times 950$)

to coalesce, and, even when the material is very thickly spread, are still discrete but very minute.

(d) The appearance of colonies on blood-agar is very similar to that on plain agar, except that they are larger and slightly brown in colour. ✓Each is surrounded by an area in which the medium has become quite clear and of a pale yellow colour, owing to the lysis of the red blood corpuscles and alteration and diffusion of the haemoglobin. The diameter of this clear area may be as much as 4 or 5 mm. Where the colonies are fairly closely associated, the adjoining zones unite and the medium in the neighbourhood

is all of the clear yellow colour. Some strains of *Str. pyogenes* produce hæmolysis on blood-agar only when cultures are incubated anaerobically and, with all strains, more marked hæmolysis is shown by deep colonies than by those on the surface of the medium.

2 In broth this *Streptococcus* usually grows in granules, which may vary in size from those only visible with the microscope to quite large flakes. The result is that the masses of organisms fall to the bottom of the tube, leaving the upper part almost or entirely clear. The addition of serum or ascitic fluid to the broth usually gives a much more abundant growth than would be obtained in its absence. Glucose also encourages growth but, on account of the acid produced, growth soon ceases and the organism dies unless the amount of glucose is restricted and the medium is well buffered.

By the growth of *Str. pyogenes* in broth of suitable composition, two hæmolyins are produced and are found free from cocci in the filtrate of such a culture. One of these lysins (O) is very rapidly inactivated in the presence of oxygen the other (S), which is produced only in broth containing serum, although very labile at body temperature, is not inactivated by oxygen. Most strains of *Str. pyogenes* produce both hæmolyins but some produce only one. Hæmolyins in blood-agar resulting from surface growth is due entirely to the S lysin: in deep culture, to either the O or S lysin or to a combination of the two. A strain which produces only O lysin will not cause any hæmolysis when spread on the surface of a blood-agar plate but will in deep culture. Antilyns can be produced by the immunisation of animals. In the case of the O lysin, a filtrate of a culture may be used as the immunising agent but, to prepare S antilysin, the actual organism itself must be introduced. The leucocidin produced by *Str. pyogenes* is probably identical with the O lysin.

Another toxin, the erythrogenic or Dick toxin, which is produced by a limited number of strains of *Str. pyogenes*, will be dealt with later.

In addition to the toxins referred to above, *Str. pyogenes* pro

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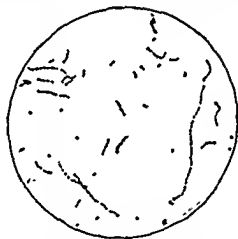


FIG. 46—*Streptococcus pyogenes* FROM BROTH CULTURE ($\times 950$).

to coalesce, and, even when the material is very thickly spread, are still discrete but very minute.

(2) The appearance of colonies on blood-agar is very similar to that on plain agar, except that they are larger and slightly brown in colour. Each is surrounded by an area in which the medium has become quite clear and of a pale yellow colour, owing to the lysis of the red blood corpuscles and alteration and diffusion of the haemoglobin. The diameter of this clear area may be as much as 4 or 5 mm. Where the colonies are fairly closely associated, the adjoining zones unite and the medium in the neighbourhood

genes A minute skin abrasion, infected with *Streptococcus pyogenes*, may be followed by a rapidly spreading cellulitis and lymphangitis and frequently by septicemia. The tendency of a streptococcal infection to spread through the tissues is probably due, not to hyaluronidase, but to the production, by virulent streptococci, of an anti-fibrinogenic substance, which prevents the formation of fibrin, and a fibrinolysin which dissolves it if already formed. These substances interfere with the efforts of the body to limit the spread of infection by the clotting of the contents of blood and lymph vessels. The invasiveness of the organism is



FIG. 47 — *Streptococcus pyogenes* IN PUS
($\times 950$)

also assisted by the absence of positive chemotaxis and by the production of leucocidin which kills polymorphonuclear leucocytes. The exudate is, therefore, inclined to be serous rather than purulent, and the body may be widely invaded before there is a satisfactory mobilisation of the phagocytes. When definite pus is produced, the prognosis is better than in cases in which the exudate contains few or no cells. *Strep. pyogenes* is a cause of pus production in serous cavities: peritonitis, empyema and meningitis are frequently due to its action. It often produces severe pharyngitis and tonsillitis, and its presence may prove a grave complica-

duces another substance, fibrinolysin, which resembles an enzyme rather than a toxin. Its action can be demonstrated by adding equal volumes of a weak solution of calcium chloride and of a broth culture of the organism to two volumes of dilute citrated plasma. A firm clot is produced within a few minutes as a result of the addition of the calcium salt. After incubation for some hours, the clot is liquefied by the fibrinolysin produced by the growth of the organism. Human plasma must be used—that of animals is not satisfactory—but not every sample of human plasma is liquefied by fibrinolysin. The coagulated plasma of a person who has recently recovered from an infection with *Str. pyogenes* often fails to liquefy owing to the antifibrinolysin which is present.

ling rapidly through the
produce hyaluronidase
with capsule formation

and the capsules of *Str. pyogenes* contain hyaluronic acid. Since hyaluronidase digests hyaluronic acid, the former substance would tend to reduce the viscosity of the medium.

S. pyogenes produces mannitol. It does not hydrolyse sodium hippurate nor does it liquefy gelatin.

The microscopical appearance of cultures depends to a very large extent on the culture medium used, the same strain of *Streptococcus pyogenes* being found in very different forms in different media. In general, in an enriched broth of correct reaction, chains of at least eight elements and frequently of twenty or thirty appear. In young cultures the chains may seem to be composed of diplococci on account of the rapid multiplication of the organisms. A broth without the addition of body fluid or one of too acid reaction may show very much shorter chains. On solid media, chain production is much less marked, and diplococci or chains of 4 or 6 cocci may be common.

Man is very susceptible to *Streptococcus pyogenes*. The lesions produced are usually less localised, tending to spread, and are more often fatal than those caused by the *Staphylococcus pyo-*

In scarlet fever, the organism tends to remain localised in the throat but its toxin is absorbed and carried by the blood to all parts of the body.

The Dick test, which is used to determine whether a person is immune or susceptible to scarlet fever, is carried out by injecting 0.2 ml. of a suspension of the organism into the skin of a person. The result follows.

by his antitoxin and no reaction occurs.

The erythrogenic toxin cannot be converted into toxoid, but active immunity to it may be produced by the subcutaneous injection of the toxin itself. A series of injections, gradually increasing from 500 to 50,000 S.T.D., usually causes the development of a sufficiently high level of immunity to protect against scarlet fever. The S.T.D. (skin test dose) is the amount of toxin

of uncomplicated scarlet fever before the introduction of modern chemo therapeutic drugs and the antibiotics.

The main reservoir of Str. pyogenes is the human throat. There it may cause tonsillitis, laryngitis, or pharyngitis, and there, as in the nose, it may exist without producing symptoms. In the latter case, the individual concerned is a carrier, and it is mainly from such carriers that the organism spreads to others to cause, in

Many well-qualified observers believe that throat infections with Str. pyogenes are responsible for acute rheumatism. There is very commonly a history of a severe tonsillitis at a period varying from a few days to 4 weeks antecedent to the attack of rheumatism. The organism can be isolated from the throats of the great majority of patients and it has been found in the heart valves of patients who have died during the acute stages of the disease.

tion in diphtheria. *Streptococcus pyogenes* is commonly found in acute bronchitis and broncho-pneumonia, usually associated with other bacteria but in some cases apparently pure. Osteomyelitis is less frequently due to streptococci than to staphylococci, but, when the former are responsible, the prognosis is very much more serious. Erysipelas is one of the results of infection of the skin by this organism. Puerperal fever is most frequently due to *Streptococcus pyogenes*. The most serious condition caused by it is a septicæmia, usually accompanied by acute endocarditis which is a common termination of many streptococcal infections. The number of organisms in the blood may be as high as 5,000 per cubic centimetre but, before the discovery of sulphonamide and penicillin, survival was very rare when the number exceeded 30. Blood cultures, in cases of suspected streptococcal septicæmia, should be incubated for a week before being regarded as negative as the cocci may grow very slowly.

✓ Some types (and particularly types 1, 2, 3, 4 and 5) of *Str. pyogenes* produce an exotoxin called the erythrogenic or Dêltoxin. The intradermal injection of a filtrate of a broth culture of one of these types causes an intense local erythema of the skin and the subcutaneous injection of a larger amount produces a condition similar in many respects to scarlet fever.

✓ When the throat of a non-immune person is invaded by one of these types, the result is an attack of scarlet fever. Immunity to this toxin must be carefully distinguished from immunity to a particular type of *Str. pyogenes*. A person who has recovered from an attack of scarlet fever due to *Str. pyogenes* of, for example, type 2, possesses immunity both to the erythrogenic toxin and to streptococci of type 2. This immunity should suffice in the future to protect him from scarlet fever, irrespective of the type of *Str. pyogenes* which may invade his throat. It should also protect him from infection by an organism of type 2 but, since the main type specific, it will not protect him against *Str. pyogenes* of types 1, 3, 4, 5, etc., any one of which may cause in him a severe attack of tonsillitis and pharyngitis but not scarlet fever.

of a joint become sensitised to some product of the streptococci : and that the disease is due to an allergic reaction of the sensitised tissues to the substance.

Other Streptococci

Many streptococci, apart from those described above, exist in a variety of habitats and may, from time to time, be isolated from the human body, particularly from the mouth, nose, throat, faeces and vagina. Among these are the lactic acid streptococci associated with the souring of milk and closely related to *Str. faecalis*. One occasional human pathogen deserves mention. This is an anaerobic streptococcus which causes a severe type of gangrene in wounds.

Str. mitis, although a commensal, may also act as a pathogen. While in the mouth it inflicts little, if any, damage. Occasional it invades the tissues, producing subacute or chronic disease with little pus production. There is good evidence that the organism very commonly enters the blood stream as a result of extraction of or other interference with a tooth. The resulting bacteræmia is usually transient. It has previously been noted that it is often found on the valve when due to rheumatic fever, and is usually highly resistant to treatment.

Str. faecalis (or the enterococcus) is associated with the intestinal tract and is invariably present in human feces. It occurs either in short chains or, more commonly, in pairs. Individual cocci are elongated, sometimes almost bacillary. The organism usually produces no alteration in blood agar. Some strains, however, produce a green colour and a few true hæmolysis. Serologically *Str. faecalis* belongs to Lancefield's D group whether it is hæmolytic or not.

In broth, *Str. faecalis* gives rise to a uniform turbidity with very little deposit. Some strains liquefy gelatin. The organism ferments glucose, lactose, and mannitol. In addition to the morphology of this organism, its most important differential characteristics are its power of growing in the presence of bile and its thermo-resistance. *Str. faecalis* is usually capable of growth at a temperature of 50° and survives exposure to 60° for half an hour. Like *Bact. coli*, the *Str. faecalis* is, in the intestine, a mere saprophytic parasite but, in the tissues, it is both pathogenic and pyogenic. It is an occasional cause of puerperal sepsis. Like *Str. mitis*, it may enter the blood-stream and cause subacute endocarditis. Unlike *Str. mitis*, this organism is usually highly resistant to penicillin.

While there is a widespread belief that chronic rheumatism is in some way, connected with streptococci, and particularly non-hæmolytic streptococci, no definite proof of the connection has yet been afforded. The most plausible theory is that the tissues

incubated. On serum or ascitic agar the colonies of a capsulated pneumococcus are somewhat larger than those of the *Strepto-*

like deposit of the bacteria may fall to the bottom of the tube

usually, inulin

Microscopically, in young cultures on suitable artificial media, the typical lance-shaped diplococci are seen, together with short chains chain formation is particularly likely to occur in broth forms in able

cultures, serum should be used as a diluent in place of water.

adverse media lity for light and air are excluded. It remains alive for a long time in the blood of an infected animal which has been dried rapidly in a desiccator

chemical disinfectants and is very rapidly killed by exposure to direct sunlight.

CHAPTER XXIV

Diplococcus pneumoniae (*Pneumococcus*)

Weichselbaum, 1886

Diplococcus pneumoniae occurs most typically as a diplococcus, each coccus being roughly oval, with one extremity pointed and the other rounded, the whole resembling a lance or the flame of a candle in shape. Usually the rounded ends of the two cocci are adjacent. Its longest diameter is about μ . It may also be seen singly, or in short chains of 4 to 6 cocci. When examined direct from the body, it is generally surrounded by a capsule, the total width of the coccus and capsule being about three times that of a naked coccus. The capsule rarely shows any indentation corresponding to the space between the two cocci.

The pneumonococcus stains well with the usual aniline dyes and is Gram positive, but dead or degenerated forms may lose the stain in Gram's method. In preparations treated with the ordinary stains the capsule does not take the colour, but can often be distinguished as an unstained halo round each pair of cocci. It may be demonstrated more satisfactorily by the methods recommended for the staining of capsules. This organism is non-motile, is without flagella and forms no spores.

Diplococcus pneumoniae is aerobic and facultatively anaerobic. In artificial media its growth is never luxuriant. It may not grow, specially when recently isolated, unless the medium has been enriched by the addition of blood or serum. The presence of glucose in the medium also promotes growth, but the organism is soon killed by the resulting acidity, unless the medium is well aerated and the glucose restricted to 0.2 per cent. The optimum temperature of culture is from 35° to 37°, and no growth occurs below 22°. Growth is encouraged by the addition of 5 to 10 per cent of carbon dioxide to the atmosphere in which cultures are

pneumococci, the latter from combination of antibody with the

a large number of serologically distinct types of pneumococci—over 30 have so far been differentiated—owing to minor chemical and, therefore, antigenic differences in the S.S.S. of these different cocci. With the exception of Type III, referred to above, these are morphologically and culturally indistinguishable. The capsule confers on a pneumococcus its virulence, its toxicity, and its type specificity. When the capsule is lost, a pneumococcus is non-virulent.

It is in antibody that the transformation takes place. That a pneumococcus, irrespective of its original type. The types are not so stable as was once believed. A pneumococcus, originally of say Type I, which has lost its capsule, may be made to adopt the capsular material of another type (e.g. II), and so be transformed into a Type II pneumococcus.

Almost all cases of true acute lobar pneumonia are due to *Diplococcus pneumoniae*, sometimes associated with other organisms (*H. influenzae*, streptococci, staphylococci, etc.) or, as more commonly happens, pure. In broncho-pneumonia of children, the pneumococcus is of commoner occurrence than any other single organism. Pneumococci are present in large numbers

in them.

While pneumonia is the chief disease caused by *Diplococcus pneumoniae*, it is by no means the only one. The organism is found

All pneumococci are dissolved by animal bile or by solutions of sodium desoxycholate. An easy method of testing this is to add 1 part of a 10 per cent. solution of sodium desoxycholate to 9 parts of an early serum broth culture. For this test the acidity of the culture must not exceed a pH of 6.6 as, in more acid solutions, bile salts are precipitated. Solution may take place almost at once or after a few minutes' heating to 37°. The dissolution of the pneumococci may be judged by the clearing of the broth or by the absence of cocci when the culture is examined with the microscope. This



FIG. 48.—PNEUMOCOCCI IN FILM OF PUS,
STAINED TO SHOW CAPSULES ($\times 950$)

... pneumococci and streptococci
... somewhat from those of other
types. They are larger, are commonly oval or spherical, and tend
to occur in chains. The large size of their colonies, which are
sticky when touched with a platinum wire, is due to their marked
capsule development.

pneumococcus is mixed with a specific antiserum, both agglutination and precipitation occur. The former results from combination of antibody with antigen present in the intact capsules of the pneumococci, the latter from combination of antibody with the

known as the precipitation reaction. The same serum has no effect on the capsule of a pneumococcus of a different type. There are a large number of serologically distinct types of pneumococci—over 30 have so far been differentiated—owing to minor chemical and, therefore, antigenic differences in the S.S.S. of these different cocci. With the exception of Type III, referred to above, these are morphologically and culturally indistinguishable. The capsule confers on a pneumococcus its virulence, its toxicity, and its type specificity. When the capsule is lost, a pneumococcus is non-virulent and no longer reacts with an antiserum prepared against it in its capsulated condition, but these

antibodies, but these. That is, the serum w
coccus, irrespective of its original type. The types are not so stable as was once believed. A pneumococcus, originally of say Type I which has lost its capsule, may be made to adopt the capsular material of another type (e.g. II), and so be transformed into a Type II pneumococcus.

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While pneumonia is the chief disease caused by *Diplococcus pneumoniae*, it is by no means the only one. The organism is found

in the fibrinous or sero-fibrinous pleurisy, so commonly associated with pneumonia, and also, sometimes accompanied by streptococci or staphylococci, in the empyema which often follows.

✓ Pneumococci
cases of pne
tion explain
the disease:
peritonitis a

and peritonitis of pneumococcal origin—may occur, especially in children, without an antecedent attack of pneumonia. Primary meningitis may be a very acute and rapidly fatal condition. The cerebro-spinal fluid may be turbid from the presence of enormous numbers of pneumococci, and may show practically no pus cells.

The most commonly occurring types of pneumococci in acute lobar pneumonia are Nos. I, II, III, IV, V, VII and VIII. Types I and II are responsible for more than half the cases. The disease due to Type III is more fatal than that caused by other types. Broncho-pneumonia and bronchitis may be due to one of the same types but, more commonly, are caused by other and less virulent types.

✓ Although pneumococci are almost constantly present in the mouth, throat, nose and bronchi, the majority found in health in those who have not been in contact with a case of pneumonia are either non-capsulated and non-virulent or belong to one of the less highly pathogenic types.

carrier, for persons who have been in contact with a case of pneumonia may be found to harbour fully virulent cocci of the same type as that causing infection in the patient without themselves acquiring the disease.

Man occupies towards *Diplococcus pneumoniae* a position mid-way between animals which are almost or completely refractory, such as rats and cats, and those which are very susceptible as, for example, mice and rabbits. Intravenous inoculation of mice and

rabbits is almost always followed by a rapidly fatal septicæmia. The same result usually succeeds the peritonitis of fibrinous or sero-fibrinous nature produced by intraperitoneal inoculation. A simple method of isolating pneumococci from sputum is by the intraperitoneal inoculation of a mouse, the organisms being recovered after death from the blood. By using a strain of correct virulence, it is possible to produce lobar pneumonia in rabbits by intratracheal inoculation. If the virulence is too high, septicæmia results, if too low broncho-pneumonia or only bronchitis. In monkeys, by the same technique, typical lobar pneumonia, resembling that of man, may regularly be produced.

..... it is easily lost
 dy fluids. The
 f capsules and
corresponds with the change from smooth to rough colony formation.

Pneumonia in man is a fatal disease, not on account of the local injury to the lung, for this is not commonly the immediate cause of death, but partly by reason of the intense toxæmia which is the cause of the terminal vaso-motor collapse, and partly owing to the invasion of the blood-stream. Before the introduction of effective chemotherapy, the fatality rate in pneumonia in patients with pneumococci in their blood was about seven times that of patients with negative blood culture.

Before the introduction of the sulphonamides, antisera were used for the treatment of pneumonia. To be effective, the antiserum had to correspond to the type of pneumococcus responsible for the attack. This led to the development of rapid methods of typing of which the most satisfactory is that of Neufeld. A loopful of sputum is mixed, on a cover glass, with two or three loopfuls of type serum and one loopful of methylene blue stain. From this a hanging drop preparation is made. If the pneumococci are of the same type as the antiserum, the capsules of the pneumococci are seen to be swollen and much more clearly defined than in a preparation containing a different type serum.

The severity and fatality, not only of pneumonia but also of

in the fibrinous or sero-fibrinous pleurisy, so commonly associated with pneumonia, and also, sometimes accompanied by streptococci or staphylococci, in the empyema which often follows. Pneumococci are commonly present in the blood stream in severe cases of pneumonia.

tion explains the fact
the disease: otitis me
peritonitis and arthrit

and peritonitis of pneumococcal origin—may occur, especially in children, without an antecedent attack of pneumonia. Primary meningitis may be a very acute and rapidly fatal condition. The cerebro-spinal fluid may be turbid from the presence of enormous numbers of pneumococci, and may show practically no pus cells.

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example, mice and rabbits. Intravenous inoculation of mice and

CHAPTER XXV

Neisseria meningitidis (*Meningococcus*, *Neisseria intracellularis*) Weichselbaum, 1887

N. meningitidis is the causal organism of ...
of
fro
ma
micro-organisms, in the early stages of very acute cases,
many may be free from cells, which may be few in number.
Most typically they are found as diplocci, the cocci being
somewhat flattened on their adjacent sides, which gives to the
individual somewhat the shape of a coffee bean.

with the ordinary aniline dyes, but it is frequently observed that
some individuals take the stain much more intensely than others.
They are Gram-negative, non-motile and do not form spores.

N. meningitidis, while not very difficult to cultivate, demands
a rather rich medium. When taken fresh from the body, no
growth may be obtained on plain agar, but on agar containing
blood, serum, or ascitic fluid, good growth occurs. The presence
of 0.5 per cent. of glucose also improves the growth. Freshly
isolated strains will not grow below 23° and only feebly at that
temperature. Optimum growth is obtained at 37°. It is a strict
aerobe.

other pneumococcal infections and especially of pneumococcal meningitis, have been markedly reduced by treatment with the sulphonamides and penicillin. These remedies are much more

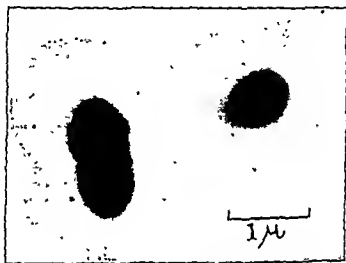


FIG. 49.—PNEUMOCOCCI SHOWING NEUFELD REACTION.
(Electron Microscope)

effective than the serum treatment which they replaced and are also more easily administered. They have the further advantage of obviating the necessity of determining the type of pneumococcus responsible for the condition.

causative organism usually belongs to Group I. Sporadic cases of the disease are more commonly due to cocci of Group II.

The chief disease produced in man is meningitis involving

within 24 hours, or may be subacute or almost chronic. The cerebro-spinal fluid is under considerable pressure, and, at a late stage of the disease, may be turbid from the large number of cells (almost entirely polymorphonuclear leucocytes) contained in it. The presence of meningococci may usually be detected by microscopic examination of the centrifuged deposit of the cerebro-spinal

and, in some cases, before symptoms of meningitis were detectable. The organism also causes chronic bacteremia, characterised by irregular fever, crops of tender, reddish spots in the skin, pain and effusion in joints and muscular pains. Meningitis may, but does not necessarily, occur in this condition. The most common situation in which the meningococcus is found is the naso-pharynx, where it produces a rhino-pharyngitis. It is there found in many persons who have not had and who do not develop

naso-pharynx of one to the naso-pharynx of the other. The method by which the organisms pass from this situation to the meninges cannot yet be regarded as certain, but transfer by the and thence to the ventricles why one man with meningitis while another does not. We can only say that it is a question of individual susceptibility or of individual resistance. We know that the proportion of meningococcal carriers is higher among contacts than

smooth, they are almost transparent. When touched with a platinum wire they are found to be rather viscid, but emulsify easily in saline. There is no hæmolysis in the neighbourhood of the colonies. Coagulated serum and coagulated egg are useful media for the cultivation of the organism. The meningococcus grows feebly in fluid media. It ferments glucose and maltose without gas production and has no action on saccharose.

Films made from cultures resemble fairly closely those prepared from cerebro-spinal fluid, but the occurrence of the diplococcal form is not quite so regular. The irregularity in size and in



FIG. 50.—MENINGOCOCCI IN PURULENT CEREbro-SPINAL FLUID

staining is even more marked, and in preparations made from cultures 48 hours old, many large, badly staining involution forms are seen.

This organism has very little resistance against adverse conditions, and cultures kept at 37° may be found to be dead in 3 or 4 days. It is rapidly killed by drying, by exposure to heat or cold or to sunlight.

By the inoculation of animals it is possible to prepare agglutinating sera. The majority of meningococci may be assigned to one of two groups, I and II. In epidemics of cerebro-spinal fever, the

Chief one of these likely to cause confusion is *N. catarrhalis*. The colonies of *N. catarrhalis* are more opaque than those of *N. meningitidis*, they have slightly irregular edges and are definitely white. The organism may grow at 23°, but often only feebly. It has no fermentative action on glucose, maltose, or saccharose. *N. catarrhalis* is frequently associated with acute and chronic inflammations of the respiratory tract. It is probably not the primary infecting agent in these conditions, but an important secondary invader. It is almost devoid of virulence for animals.

among non-contacts, and that it rises in the winter and spring months (at which times epidemics are commonest), particularly among people living under conditions of overcrowding. The duration of the carrier state is not usually long—a few weeks—but it may persist obstinately for months. Attempts to control epidemics of cerebro-spinal fever by the isolation of carriers were often made in the past but were rarely successful. They were almost farcical when, as sometimes happened, the carrier rate exceeded 50 per cent. The prevention of overcrowding, particularly in dormitories and barracks, were of value but it is probable that, in future, carriers will be treated with sulphonamide which, in the majority of cases, brings the condition to an end within 72 hours.

The meningococcus possesses but a low grade of virulence for animals. By intracerebral or intrathecal injection of young cultures in certain types of monkeys an acute meningitis has been produced. Intravenous or intraperitoneal inoculation of mice or guinea-pigs with highly virulent strains gives rise to a fatal

gococ
have 1
living cocci may be very little different. This points to the existence of a powerful endotoxin in the meningococci. No exotoxin has been demonstrated in cultures.

For many years the only specific treatment for cerebro-spinal fever was with anti-meningococcal serum, prepared by the immunisation of horses. The antiserum which had to be group- or type-specific, was administered either intrathecally or intravenously. The potency of different batches of serum varied greatly. This method of treatment has been discarded, without regret, in favour of sulphonamides. Since the meningococcus is normally penicillin-sensitive, this antibiotic may be used as an alternative to sulphonamide.

The identification of *N. meningitidis* in the cerebro-spinal fluid

presents no difficulty. In cultures taken from the naso-pharynx, considerable trouble is experienced, for a number of other Gram-negative diplococci are frequently found in that situation. The chief one of these likely to cause confusion is N. catarrhalis. The colonies of N. catarrhalis are more opaque than those of N. meningitidis, they have slightly irregular edges and are definitely white. The organism may grow at 23°, but often only feebly. It has no fermentative action on glucose, maltose, or saccharose. N. catarrhalis is frequently associated with acute and chronic inflammations of the respiratory tract. It is probably not the primary infecting agent in these conditions, but an important secondary invader. It is almost devoid of virulence for animals.

CHAPTER XXVI

Neisseria gonorrhææ (*Gonococcus*) Neisser, 1879

N. gonorrhææ is an organism which resembles *N. meningitidis* very closely in microscopic appearance and in cultural characteristics. It is most commonly found in the body as a diplococcus, the size of a pair of cocci together being about 1.5μ by 0.8μ . The cocci are either flat or, more usually, somewhat concave on their adjacent sides: in the latter case a small oval unstained area is visible between them. They have been described as resembling

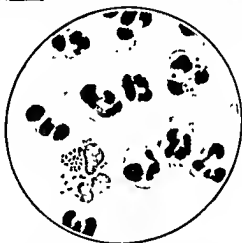


FIG. 51.—GONOCOCCI IN PUS ($\times 950$).

a pair of kidneys or beans placed together with their concave borders in apposition. In stained films of the pus obtained from the urethra in acute gonorrhæa, the great majority of the cocci are seen to lie within leucocytes. As many as one hundred cocci may occasionally be seen within a single pus cell. It is remarkable that often, so far as can be determined by microscopical examination, neither the cocci nor the pus cells have been injured by this

invasion, both taking the stain normally. The nucleus is never invaded by the cocci. In the very early stages of gonorrhœa, when the discharge is mucoid rather than purulent, very many gonococci may be seen outside pus cells, and a similar picture may be obtained in an old standing case of gleet, except that in this condition the cocci may be very scanty. The gonococcus takes the common aniline stains well, but is Gram negative. For diagnostic purposes, Gram's stain should always be used but, if the number of gonococci be few, they may easily be overlooked. By using Pappenheim's stain as the counter-stain in Gram's method, the advantage of a contrast in colour between the cocci (red) and the cells (blue) is secured without sacrificing the diagnostic value of Gram's method.

The most satisfactory medium for its growth is soft, boiled blood-agar. This is made by mixing melted nutrient agar with an equal volume of broth and adding to the mixture 5 per cent. of citrated blood. The tubes or flasks containing the medium are kept in boiling water for a few minutes before the medium is allowed to cool and solidify in plates or, sloped, in tubes. It is essential to use the medium soon after its preparation and before its surface has become dry. The gonococcus is aerobic, but growth takes place best when cultures are incubated in an atmosphere containing 10 per cent. carbon dioxide. Precautions should be taken to prevent the drying of the medium during incubation. A temperature of 35° to 36° is better for growing the gonococcus than the customary 37°.

Colonies usually appear within 24 hours, but may be delayed for 48 hours or even longer. The colonies vary in size, but are rarely more than 2 mm. in diameter and are usually smaller. They are semi-transparent and of a greyish-white colour. In shape the colonies are round and their margins are often scalloped or undulating. Later the centre of the colony becomes rather more opaque and granular. The colonies are usually discrete and have a somewhat viscid consistency.

Since their colonies are small and almost transparent, it may be difficult to detect by culture the presence of a few gonococci

in material which contained large numbers of other and more robust bacteria. The following reagent is of use after the culture has been washed in distilled water and is 1 per cent. watery potassium bichromate in hydrochloric hydrochloride. When acted upon by this reagent, the colonies of V. gonorrhæe become first pink and later magenta owing to the presence in them of oxidase (produced by the growth of the organism). The reaction is not specific for the gonococcus, being given also by other neisseriæ, but these rarely occur in situations in which the gonococcus is likely to be found. The reagent does not kill the organism so quickly as to prevent subcultures from a

h is obtained, the medium becomes turbid. After 24 hours the medium becomes clear and settles to the bottom of the tube and partly owing to autolysis. Occasionally a pellicle forms on the surface. The gonococcus ferments glucose but not maltose or saccharose. The microscopic appearance of gonococci in culture resembles that seen in direct films, but there is much greater regularity in size, swollen and badly staining degenerated cocci being present, and the diplococcal form is accompanied by single cocci or tetrads.

The gonococcus has very slight powers of resistance. It dies in culture in a few days at 37°, and even more rapidly at air temperature or in the refrigerator. It is killed by exposure to a temperature of 42° for some hours. Desiccation and exposure to sunlight or to weak disinfectants kill it rapidly. It has, however, been found to survive as long as several weeks when dried in a thick layer of pus.

By the inoculation of animals agglutinating sera may be produced. There are a considerable number of distinct serological types of gonococci. This fact has to be allowed for in making use of complement fixation tests for diagnosis and in using vaccines for treatment.

Man is the only animal naturally infected by gonococci. Infection is, on account of the delicacy of the organism, almost

always direct from individual to individual. The gonococcus attacks chiefly the urethra, both in the male and female, producing an acute catarrh. The cocci quickly penetrate the surface, passing between the epithelial cells which are loosened and desquamated, and invade the tissues as far as the superficial layers of the sub-mucous connective tissue. There is an energetic emigration of polymorphonuclear leucocytes, and the discharge, at first mucoid, soon becomes purulent. The disease spreads and, in the male, if untreated, the prostate may be involved and occasionally the bladder. Orchitis and inflammation of the cord and epididymis are also of fairly frequent occurrence. In the female the urethra

generally venereal, but is due to the use in common of utensils or clothes, especially in children's hospitals and schools in which the condition may become endemic. The disease in the woman often involves the glands of Bartholin and, more rarely, the The gonococcus is a common uent sterility and of localised sex, the mucous membrane of the rectum and anus may be involved. Gonococcal conjunctivitis may occur at any age, but particularly in the new-born, from infection during parturition. The resulting ophthalmia neonatorum, if neglected, may cause blindness. Gonococcal septicæmia, with or without endocarditis, occurs as a complication of gonorrhœa, as also does arthritis

The gonococcus is almost without pathogenicity for the lower animals.

very little, if any, immunity, may exist in a quiescent scanty or indeed absent.

A person in this condition may infect another in coitus, or the disease may suddenly become active in himself, often in response to some stimulus such as an alcoholic debauch.

The bacteriological diagnosis of acute gonorrhœa in the male

is not difficult, as the microscopic picture in films of pus from the urethra is so characteristic. Gram's method of staining, it should be emphasised, is the only reliable one for diagnostic purposes. A diagnosis can never be given in any case unless typical grouping of Gram negative diplococci is seen within a cell. In the female, diagnosis is less easy. Pus from the urethra, cervix, gland of Bartholin or clitoris should be obtained: vaginal swabs are practically useless. Since other Gram negative cocci are frequently found in the vagina, in addition to satisfying the criteria mentioned above, cultures should be made. When pus is scanty its amount may be increased, and also the number of gonococci, in various ways. The chief of these are excess of alcohol, injection of silver nitrate solution into the urethra and the use of a vaccine in sufficient dose to obtain a marked local reaction. In other parts of the body the diagnosis is even more difficult, on account of the possible presence of other neisseriæ. Cultures should be obtained and the gonococcus identified by its various characteristics, including its fermentation of glucose only. Apart from microscopic and cultural methods, a diagnosis of gonorrhœa may sometimes be made by the complement fixation test. This is uncertain in acute cases, but in the late complications of the disease, such as arthritis, it is of considerable value. On account of the many types of gonococci it is essential that the antigen should be polyvalent.

The treatment of gonorrhœa has been revolutionised by the introduction of the sulphonamides and penicillin, to the action of both of which normal strains are highly susceptible. As a result of inadequate and irregular treatment with sulphonamides, resistant strains of the organism have developed. For the treatment of patients suffering from infection with one of these strains, penicillin should be used. Resistance to penicillin may also develop for the same reasons and we feel it only right to warn every doctor having the care of a patient suffering from gonorrhœa that it is his duty to treat that patient, whether with sulphonamide or penicillin, adequately or not at all.

Close Examination.
iii → Gram positive, aerobic or facultative
anaerobic, sporing rods which occur in chains, they are mostly
saprophytic, only one pathogenic is
the Anthrax bacillus, being known
CHAPTER XXVII

Bacillus anthracis **Koch, 1876**

B. anthracis shows considerable variations in size, being usually 5-10 μ by 1-2 μ . It is found, in the lesions which it produces in animals and man, singly, in pairs, or in short chains, which rarely consist of more than five individual bacilli. Under natural conditions it is surrounded by a capsule. The adjacent ends of bacilli in chains appear either to be sharply cut-across or slightly dimpled. The result of the latter form is that an oval unstained area separates the bacilli. The whole chain. The organism is seen in preparations made from removed from the body. The bacillus stains easily and is Gram positive.

The anthrax bacillus is aerobic and facultatively anaerobic. The organism grows well on all ordinary media at temperatures between 19° and 40°. Growth ceases below 14° and above 44°.

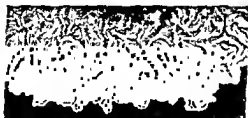
The colonies on agar are of a greyish white or cream colour. They have a rather solid opaque centre with an irregular, wavy, less opaque margin. When more closely examined with a lens the colony is found to be made up of tangled threads, somewhat resembling matted locks of hair or a tuft of cotton wool. The colony is so characteristic that, once seen, it can readily be recognised again. With the low power of the microscope it is found that the strands or threads which make up the colony are each

distance is some-
outh shows the

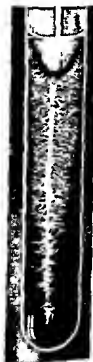
upper part of the medium than below, the whole somewhat

bottom of the tube, leaving the upper part of the broth almost clear. The bacillus ferments glucose, maltose, and saccharose without gas production.

The microscopic appearance of anthrax bacilli in culture is different in many respects from that of bacilli removed from an animal's body. In artificial media very long chains of bacilli occur. Capsules are not formed unless the medium is fluid serum and, even in this, their presence is not constant.



(Tangled Threads colony)
FIG. 52.—EDGE OF COLONY OF *B. anthracis*
ON AGAR (X 75).



(Inverted Fir-Tree)
FIG. 53.—*B. anthracis* IN
GELATIN (X 1)

After a few days' growth, or even in 24 hours in some cases, a small refractile spot is to be seen in the centre of many of the bacilli when they are examined unstained. In stained preparations this spot may appear to be at first a less intensely stained part of the bacillus. After a short time its size increases, until it is seen to be a definitely outlined, unstained body situated at about the centre of the bacillus. Gradually the bacillus disintegrates and

this body, now the fully formed, oval spore, is liberated. The spore is uncoloured by ordinary stains, but may be demonstrated by one of the special spore stains. As has already been mentioned, spores are never found in the body of an animal during life.

Further facts concerning the spores of *B. anthracis* are as follows:—
 1. Spores are not produced in a culture of *B. anthracis* at 14°.

2. In fact, by prolonged cultivation of the bacilli at 42° to 43°, or by growing on a medium containing phenol, a strain is produced which may never regain the power to produce spores. The explanation of these isolated facts concerning the spore

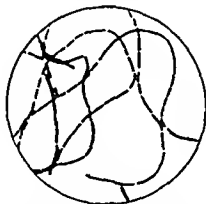


FIG. 54.—*B. anthracis* FROM AGAR CULTURE ($\times 750$)

production of anthrax bacilli is unknown, but the facts themselves are of considerable practical importance in dealing with the disease

The resistance of the vegetative form of anthrax bacilli is less

ture of 140° may be necessary to kill them, but when wet 10 minutes boiling is sufficient. The power of resistance of different strains against disinfectants is very variable. Growth has occurred

from spores which had been in contact with a 5 per cent. solution of phenol for 14 days. Formalin is one of the most potent disinfectants against anthrax spores, and is largely employed in the treatment of infected animal products such as wool and hides.

The natural disease affects herbivora—chiefly sheep and cattle—in which the mortality may be as high as 80 per cent. Horses, goats, and pigs are less commonly attacked. Elephants are susceptible. The most marked enlargement of has been given colour, is very numbers of bacilli. The other bac are rea chi the

rarely causes infection. It has been suggested that The consumption of food which inflicts damage to the mucous membrane is essential. If, for example, thorns were ingested with anthrax spores, the damage inflicted by the thorns might be sufficient to

As has been said above, spores are not found in the body, but before death there are commonly bloody discharges from the mouth, nostrils or anus and these contain anthrax bacilli. On the ground the bacilli may assume spore form, and so the pasture is infected, and may remain in this condition for many years. If the animal's body is deeply buried unopened, the bacilli in it, being without oxygen, cannot spore and, in consequence, rapidly die. Only where a post-mortem examination has been performed is there any great danger of spreading the disease from a dead body. Animals may also be affected with the cutaneous form of anthrax

similar to that of man, but this variety is much less common than the preceding.

Man occupies a position as regards resistance higher than such animals as sheep and cattle, but lower than dogs, cats and birds, which are relatively insusceptible. He may be infected by the cutaneous, respiratory or intestinal routes. Skin infection commonly occurs in farmers, veterinary surgeons, butchers, or those whose work is in connection with hides, hair or wool, but infection has also resulted from the use of shaving brushes, the bristles of which had come from infected animals. The spores or bacilli gain entrance through cuts or abrasions or possibly by way of the hair follicles. The face and neck, hands, arms or back are the usual sites for the characteristic lesion, which is known as the Malignant Pustule. From 1 to 3 days after infection a small

increases, the lymphatic vessels and neighbouring glands become swollen and the blood stream is invaded. The fatality rate of the untreated disease in man is from 5 to 15 per cent. Once a definite

ulcers. The surrounding tissues are congested and oedematous and the bronchial and mediastinal lymphatic glands are enormously enlarged and engorged. The lung tissue itself shows similar changes frequently present. Pleural effusion and a state of general sepsis are common in the absence of treatment with penicillin, occurs in practically all cases.

The intestinal form, which is rare in man, may be due to the

the swallowing of
t by hæmorrhagic
the appearances,

in the later stages, resemble fairly closely those found in the disease in sheep.

The smaller laboratory animals—mice, guinea-pigs and rabbits—are susceptible, in the order named, to anthrax infection as a result of subcutaneous inoculation. This fact allows us to judge the virulence of a given strain of anthrax bacillus with considerable accuracy. If fully virulent, it will kill all three animals; if of moderate virulence, mice and guinea-pigs but not rabbits will



FIG. 55.—*B. anthracis* IN SECTION OF KIDNEY OF INFECTED GUINEA-PIG (X 400).

succumb; while if the virulence is low only the mouse will die. A strain almost or completely devoid of virulence fails to cause the death of any of the three animals. As a result of subcutaneous inoculation of fully virulent bacilli, the animal dies within 72

in large numbers in the heart's blood.

The precise cause of death in anthrax is as yet uncertain. There may be a mechanical factor from capillary blockage, but this cannot be the chief one. The disease has the appearance of an

~~intense toxin~~ but ~~forming~~ other endotoxins or exotoxins, being

slowly than with most other organisms. A strain which has lost its power of producing capsules and the colonies of which are relatively smooth has usually also lost its virulence. Virulence can be lowered fairly rapidly in a number of ways. Pasteur, by culturing the bacilli at a temperature of 42° to 43° , produced a strain of non-virulent bacilli. Loss of virulence is also attained by the addition of small amounts of phenol or other antiseptic to the culture medium. Virulence can be exalted by passage through susceptible animals.

Prophylaxis, so far as man is concerned, is a matter chiefly of industrial hygiene. In the case of the herbivorous animals, ordinary hygiene is impotent, since the sterilisation of infected pastures is impossible. Pasteur immunised sheep and cattle with living cultures of the bacilli the virulence of which had been reduced by cultivation at 42° . In about 1 per cent. of the inoculated animals death from anthrax occurred; but this was preferable to the very high mortality from the natural disease, at that time widespread throughout France. The immunity thus produced lasts about 1 year.

The laboratory confirmation of a diagnosis of malignant pustule presents no difficulties if material from the pustule is obtained at an early stage, as *B. anthracis* can easily be found in microscopical preparations and can be isolated culturally. In the later stages, however, the bacilli may not be found either microscopically, culturally, or by animal inoculation, and it may not be possible to confirm the clinical diagnosis. In the case of respiratory or intestinal anthrax it is essential to isolate the bacillus, in the one case from the sputum and in the other from the faeces, and to prove its pathogenicity before making a diagnosis. It should be recollected that, since *B. anthracis* does not spore in the body, it is wrong to heat the material examined, as by doing so any anthrax bacilli will be destroyed.

Where the case clinically resembles anthrax—especially where the patient's occupation has rendered him liable to infection, or where large Gram-positive bacilli are found microscopically in a suspicious lesion—treatment should be instituted without waiting for the final conclusions of the bacteriologist.

... .. compared from the
 s employed for
 were obtained
 with sulphonamides but now penicillin is the treatment of choice.

The isolation and identification of *B. anthracis* in hair or wool suspected of being infected is difficult. The material should be washed in sterile saline. One half of the washings may be heated to 65° for 5 minutes to kill non-sporing bacteria and the other half left unheated. From these two parts, used in various dilutions, a number of agar plates are made. Sub-cultures are made from suspected colonies and, if these are of bacteria microscopically resembling *B. anthracis*, the pathogenicity test may be applied. This is always necessary, as hair and wool may contain many bacteria closely resembling anthrax bacilli but which are not pathogenic.

non-capsulated, Motile, → *B. subtilis* (Hay Bacillus)

✓ *B. subtilis*, sometimes known as the hay bacillus, usually measures 2 to 3 μ by 0.8 μ , but longer forms and even filaments occur. It occurs singly or in short chains. It is Gram positive. Spores, which are situated near the centre of the bacilli, are produced.

... .. but formerly

form produces on agar, rough, wrinkled, opaque and mucous colonies; the capsulated form smooth, soft, mucoid, translucent, non-adherent colonies. A pellicle forms on the surface of broth. It ferments glucose, maltose, saccharose and mannitol producing acid without gas. It liquefies gelatin.

B. subtilis is found in soil, dust, decomposing organic matter

and, occasionally, in the fæces. It is commonly present in the hay and straw used for packing glassware which, as the spores are very resistant to heat, makes obvious the necessity of thorough sterilisation before the apparatus is used in the laboratory. The mucoid variant (*B. mesentericus*) is responsible for the ropiness occasionally occurring in bread.

CHAPTER XXVIII

Corynebacterium diphtheriae Klebs and Löffler, 1883

C. diphtheriae, in preparations made from cultures on serum medium, is a straight or slightly curved bacillus. Its average size is 3 to 5 μ by 0.3 to 0.4 μ ; but great variations in size are met with, depending partly on the strain and partly on the culture medium used. It stains well, but usually strain

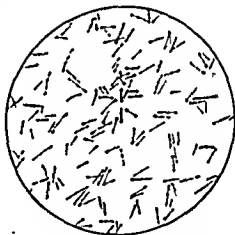


FIG. 56—*C. diphtheriae* FROM SERUM CULTURE ($\times 800$).

culture resist decolorisation less well than those lately isolated. The bacilli are rarely of uniform diameter throughout their length; most commonly one or both ends are somewhat wider than the central part, producing a "club" effect. On the other hand, certain individuals taper towards the ends, the central part being widest. Although some strains of diphtheria bacilli stain uniformly, it is more common to find an arrangement of alternately darkly and lightly staining parts. This gives to the bacillus

observed with Löffler's alkaline methylene blue stain. Frequently at the ends or more rarely, also, along oval bodies, the staining methods h Neisser's method the granules are stained blue and the rest of the bacilli brown. With an old, polychromatic methylene blue stain the

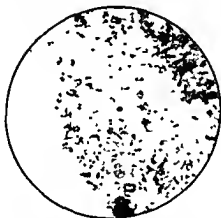


FIG 57.—*C. diphtheriæ* IN FALSE MEMBRANE (X 950)

granules appear of a reddish violet colour. In addition to these characteristics, certain other points which may assist in identifying the organism should be mentioned. There is, in a pure culture, a marked lack of uniformity in size, morphology and staining peculiarities in the individual bacilli. Long and short forms, uniformly staining and granular bacilli are seen lying side by side. There is also a rather characteristic grouping of the bacilli which occur usually in pairs. The two organisms, however, are scarcely ever arranged end to end, but most commonly close together and parallel, or with one end of one in contact with an end of the other, the two lying at an acute angle.

CHAPTER XXVIII

Corynebacterium diphtheriae Klebs and Löffler, 1883

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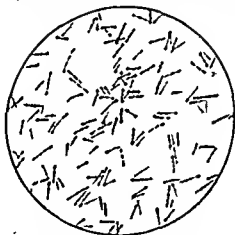
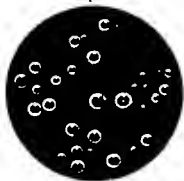


FIG. 56—*C. diphtheriae* FROM SERUM CULTURE ($\times 800$).

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1



2



3 Daisy Head Colony.



4



5



6

Blood Tellurite medium
C DIPHTHERIA

1 Intermedius group on McLeod's medium

2 Vitus group on McLeod's medium.

3 Graves group on McLeod's

4 Film stained by Gram's method

5 Film stained with methylene blue.

6 Film stained by Neisser's

The above description applies chiefly to *C. diphtheria* as it occurs in culture on serum medium. Films prepared from agar cultures show bacilli which stain less typically and in which the development of granules is less marked. After 2 or 3 days' culture

scattered irregularly, or in small groups, chiefly towards the surface. They usually are somewhat shorter, stain more uniformly and show less tendency to produce "clubs" than do bacilli from cultures. Neisser's stain may fail to demonstrate metachromatic granules, or may succeed in only a small proportion of the bacilli.

The diphtheria bacillus is aerobic and facultatively anaerobic. It is easy to contain choice.

colonies appear in less than 12 hours, that is more rapidly than the majority of other bacteria likely to occur about the throat. The result of culturing a throat swab on serum is, therefore, to increase the proportion of diphtheria bacilli to other organisms. The bacillus may grow feebly on plain agar when freshly isolated, but in later cultures grows well. Primary cultures may always be obtained on blood-agar or agar containing serum or ascitic fluid.

It has long been known that different strains of *C. diphtheria* present considerable differences in cultural characteristics. The work of McLeod and others being due to the occurrence —Gravis, Intermedius and

as types, but since a large number of types may be differentiated by serological means, we prefer to describe them as varieties. At first, for differentiation, reliance was placed on the form of colony produced on a special blood-tellurite medium, but this is now supplemented by other differential characteristics. The Gravis variety, on the special medium, produces large, grey or grey-black, daisy head colonies; in broth it produces a heavy pellicle and granular deposit with the bulk of the fluid clear; it ferments starch

and is not usually hæmolytic. The intermediate variety on the

without clouding or surface growth; it does not ferment starch and is never hæmolytic. The bacilli are almost entirely of the barred form with poor development of metachromatic granules.

ment starch and is hæmolytic. Several serological types of each of the three varieties occur.

✓ All true diphtheria bacilli ferment glucose and the majority maltose, but none ferment saccharose.

Diphtheria bacilli have much greater powers of resistance than the majority of non-sporing bacteria. They may remain alive in dried false membrane for several months at room temperature, or for 1 hour at 98°. When moist, however, an exposure to 60° kills them in a few minutes.

firmly attached to it, are the remains of the mucous membrane of the part, many of the cells of which have undergone necrosis. The local disease is frequently made more serious by the presence of other bacteria, particularly Streptococci and Staphylococci. A false membrane may be found in other parts of the body, such as the conjunctiva, vulva, vagina, and in wounds, but much more rarely than in the respiratory tract. In young children, a blood-stained discharge from the nostrils is frequently due to infection with the diphtheria bacillus.

The false membrane may cause death of the patient from obstruction to respiration, especially when situated in the larynx, but death is much more commonly due to the severe systemic

theria is grown in the form of a pellicle on the surface of broth of suitable composition until the concentration of toxin in the broth reaches a maximum, which usually occurs in 8 to 10 days. The broth is then filtered through a porcelain filter. The toxic filtrate so obtained is usually spoken of as "toxin", although toxin is only one of the substances contained in it.

state, it is much less easily injured by heat. It retains its potency for several months if kept in sealed tubes at a low temperature.

A typical toxic filtrate may have values similar to the following:

M.L.D.	.	.	.	0.002 ml.
M.R.D.	.	.	.	0.000002 ml.
L ₊ dose	.	.	.	0.180 ml.
L _r dose.	.	.	.	0.175 ml.
L _f dose	.	.	.	0.155 ml.

The M.L.D. (minimum lethal dose) is the smallest amount which kills a guinea-pig of 250g. weight in 4 days after subcutaneous inoculation.

The M.R.D. (minimum reacting dose) is the smallest amount which, injected intradermally into a guinea-pig, causes a red flush 5 mm. in diameter within 36 hours.

The L₊ dose is the amount of filtrate which, injected subcutaneously into a 250 g. guinea-pig together with 1 unit of antitoxin, kills on the fourth day.

The L_r dose is the amount which, injected intradermally into a guinea-pig together with 1 unit of antitoxin, causes a red flush 5 mm. in diameter.

The L_f dose is the amount which, when mixed with 1 unit of antitoxin, flocculates more rapidly than does any other volume with the same amount of antitoxin.

One attack of diphtheria usually protects the patient against the disease for life as a result of the development of antitoxin. A considerable percentage of people, however, who have never suffered from diphtheria do not acquire it when exposed to infec-

disturbance caused by the disease. Although a few diphtheria bacilli may be found in the internal organs, such as the spleen, there is never, in this disease, a widespread infection or septicæmia. The remote effects are due entirely to the virulent toxin formed by the growth of the bacilli. To this toxin are to be

seen. The marked lowering in the blood pressure in the disease is probably to be attributed to the effects of the toxin on the adrenal glands.

In addition to man, the rabbit and guinea-pig, the ox, horse, cat, dog and small birds are susceptible to the effects of the bacilli or their toxin. The mouse and rat are relatively immune. These animals rarely, if ever, develop the disease naturally; but in the rabbit, for example, a false membrane similar to that in man may be produced by inoculation if the mucous membrane be previously injured by scarification. The subcutaneous inoculation of a guinea-pig with a sufficient amount of a virulent culture of bacilli produces death in 36 to 72 hours. The local lesion is a small greyish area of necrosis with some fibrinous exudation; surrounding this is an extensive zone of œdema with congestion and occasionally small hæmorrhages. The neighbouring lymph glands are swollen, œdematous and congested. The internal organs are usually congested. This is most marked in the adrenals, which are often hæmorrhagic. The epithelium of the tubules of the kidney and the cells of the liver exhibit cloudy swelling. Serous effusions may occur in the body cavities, and hæmorrhages in the serous membranes. These effects are due almost entirely to the toxin, since there is little invasion of the animal's body by the bacilli. Indeed, the injection of toxin free from bacilli produces almost the same effects. If a smaller dose is administered, death may not occur c
mencin

For ; a potent
toxin. To obtain this, an actively toxigenic strain of *C. diph-*

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M.L.D.	. . .	0 002 ml.
M.R.D.	. . .	0 000002 ml.
L ₊ dose	. . .	0 180 ml.
L _r dose.	. . .	0·175 ml.
L _r dose	. . .	0 155 ml.

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In addition to man, the rabbit and guinea-pig, the ox, horse, cat, dog and small birds are susceptible to the effects of the bacilli or their toxin. The mouse and rat are relatively immune. These animals rarely, if ever, develop the disease naturally; but in the rabbit, for example, a false membrane similar to that in man may be produced by inoculation if the mucous membrane be previously injured by scarification. The subcutaneous inoculation of a guinea-pig with a sufficient amount of a virulent culture of bacilli produces death in 36 to 72 hours. The local lesion is a small greyish area of necrosis with some fibrinous exudation; surrounding this is an extensive zone of œdema with congestion and occasionally small hæmorrhages. The neighbouring lymph glands are swollen, œdematous and congested. The internal organs are usually congested. This is most marked in the adrenals, which are often hæmorrhagic. The epithelium of the tubules of the kidney and the cells of the liver exhibit cloudy swelling. Serous effusions may occur in the body cavities, and hæmorrhages in the serous membranes. These effects are due almost entirely to the toxin, since there is little invasion of the animal's body by the bacilli. Indeed, the injection of toxin free from bacilli produces almost the same effects. If a smaller dose is administered, death may not occur or may be greatly delayed. A progressive paralysis, commencing in the hind legs, appears in from 2 to 3 weeks.

For the preparation of antitoxin the first essential is a potent toxin. To obtain this, an actively toxigenic strain of C. diph-

theria is grown in the form of a pellicle on the surface of broth of suitable composition until the concentration of toxin in the broth reaches a maximum, which usually occurs in 8 to 10 days. The broth is then filtered through a porcelain filter. The toxic filtrate so obtained is usually spoken of as "toxin", although toxin is only one of the substances contained in it.

Diphtheria toxin is relatively unstable, especially when exposed to light and air. Exposure to a temperature of 58° for a few hours or 100° for a few minutes completely destroys it but, in the dry state, it is much less easily injured by heat. It retains its potency for several months if kept in sealed tubes at a low temperature.

A typical toxic filtrate may have values similar to the following:

M.L.D.	.	.	.	0 002 ml.
M.R.D.	.	.	.	0 000002 ml.
L ₊ dose	.	.	.	0 180 ml.
L _r dose.	.	.	.	0 175 ml.
L _f dose	.	.	.	0 155 ml.

The M.L.D. (minimum lethal dose) is the smallest amount which kills a guinea-pig of 250g. weight in 4 days after subcutaneous inoculation.

The M.R.D. (minimum reacting dose) is the smallest amount which, injected intradermally into a guinea-pig, causes a red flush 5 mm. in diameter within 36 hours.

The L₊ dose is the amount of filtrate which, injected subcutaneously into a 250 g. guinea-pig together with 1 unit of antitoxin, kills on the fourth day.

The L_r dose is the amount which, injected intradermally into a guinea-pig together with 1 unit of antitoxin, causes a red flush 5 mm in diameter.

The L_f dose is the amount which, when mixed with 1 unit of antitoxin, flocculates more rapidly than does any other volume with the same amount of antitoxin.

One attack of diphtheria usually protects the patient against the disease for life as a result of the development of antitoxin. A considerable percentage of people, however, who have never suffered from diphtheria do not acquire it when exposed to infec-

disturbance caused by the disease. Although a few diphtheria bacilli may be found in the internal organs, such as the spleen, there is never, in this disease, a widespread infection or septicæmia. The remote effects are due entirely to the virulent toxin formed by the growth of the bacilli. To this toxin are to be ascribed the injury the occurrence of

were frequent in the pre-antitoxin era and are still occasionally seen. The marked lowering in the blood pressure in the disease is probably to be attributed to the effects of the toxin on the adrenal glands.

In addition to man, the rabbit and guinea-pig, the ox, horse, cat, dog and small birds are susceptible to the effects of the bacilli or their toxin. The mouse and rat are relatively immune. These animals rarely, if ever, develop the disease naturally; but in the rabbit, for example, a false membrane similar to that in man may be produced by inoculation if the mucous membrane be previously injured by scarification. The subcutaneous inoculation of a guinea-pig with a sufficient amount of a virulent culture of bacilli produces death in 36 to 72 hours. The local lesion is a small greyish area of necrosis with some fibrinous exudation; surrounding this is an extensive

occasionally small hæmorrhages. The internal organs are swollen, œdematous and congested. The internal organs are usually congested. This is most marked in the adrenals, which are often hæmorrhagic. The epithelium of the tubules of the kidney and the cells of the liver exhibit cloudy swelling. Serous effusions may occur in the body cavities, and hæmorrhages in the serous membranes. These effects are due almost entirely to the toxin, since there is little invasion of the animal's body by the bacilli. Indeed, the injection of toxin free from bacilli produces almost the same effects. If a smaller dose is administered, death may not occur or may be greatly delayed. A progressive paralysis, commencing in the hind legs, appears in from 2 to 3 weeks.

For the preparation of antitoxin the first essential is a potent toxin. To obtain this, an actively toxigenic strain of C. diph-

which should be incubated for 12 to 18 hours. Films are prepared by taking sweeps from this tube, and are stained by Löffler's alkaline methylene blue, by Gram's and by Neisser's methods. As a routine, the author prefers Löffler's stain, by which the morphology of the bacillus is most clearly seen; but some bacteriologists prefer Gram's stain. Occasionally some of the diphtheroid bacilli may be mistaken for diphtheria bacillus. Atypical strains of the two may have a close resemblance to one another. It is always found, however, that, with increased experience, the number of cases in which any doubt exists greatly diminishes. In the case of a throat condition which is clinically suspicious, one is justified in reporting as a diphtheria bacillus any organism which has typical cultural and microscopical appearance. In the majority of cases the method outlined above is satisfactory but occasionally, especially when the swab was taken from a convalescent case before release, despite the selective action of solidified serum on the growth of *C. diphtheriæ*, the organism, although present in the throat, may not be found in films prepared from the serum culture. For this reason it is strongly recommended that two cultures should always be made from swabs from the throat and nose—one on serum and the other on a medium containing tellurite. The older tellurite media, such as that of Horgan and Marshall, while having the advantage of being almost selective for *C. diphtheriæ* and other corynebacteria, have two disadvantages. The first is that *C. diphtheriæ* grows so slowly on them that, in order to obtain recognisable colonies, incubation must be prolonged for 48 hours. The second is that, on these media, the morphology of the organisms is so atypical that, for their microscopic identification, they must be subcultured on serum. Both these lead to considerable delay in reporting the results of a throat swab. The newer tellurite media, and particularly Hoyle's, have neither of these disadvantages. Suspicious colonies appear within 24 hours and films can be prepared directly from the medium. A further advantage of Hoyle's

tion. They may even become carriers of virulent diphtheria bacilli without any apparent ill effects. The bacilli are in their throats but the disease does not develop. The explanation is the presence in the serum of such persons of a small amount of anti-toxin. Whether a person exposed to infection does or does not develop diphtheria depends very largely on whether or not anti-toxin is present in his blood. This can be discovered by the Schick test. A small amount ($1/50$ of a guinea-pig M.L.D.) of toxin is injected intradermally. If there is no reaction or only a very small amount of infiltration, the reaction is negative. The skin at the site of inoculation shows a bright red areola a few centimeters in diameter. It remains intense for more than a week, when it gradually passes off, but a brownish discoloration may remain for some weeks: this is a positive result. Readings are commonly made after 24 or 48 hours but the best time is probably from 5 to 7 days after injection. The amount of toxin used in the test was selected as likely to give a negative result in persons with more than $1/30$ of a unit of antitoxin per ml. in their sera, but recent work shows that an amount as little as $1/250$ of a unit may give a positive reaction. is not always sufficient faintest reaction should be considered as positive. In making the test, it is always necessary to inoculate the other arm with the same amount of toxic filtrate which has been heated to 75° to destroy its toxin. This control reveals a pseudo-reaction which is non-specific, and is due either to the protein substances of the broth or to products of the growth of the organism, other than toxin. In a pseudo-reaction, both the site of the toxin and of the control injection exhibit a certain amount of infiltration and congestion. The Schick reaction is very useful, as it enables one to judge who are immune and whom it would be advisable to inoculate in order to confer on them either passive or active immunity.

For the bacteriological diagnosis of diphtheria, it is necessary to make a culture of the bacteria collected on a throat swab. The swab should be rubbed gently over the surface of a serum tube

nose. If one of these is tested as described, both animals die, but neither shows typical post-mortem signs of diphtheria toxæmia. When several strains are to be tested for virulence, the intradermal method may be used. A suspension of the organism is made which is just opalescent (about 500 millions per ml). The hair is removed from the flanks of two white guinea-pigs, one of which has previously been protected by an injection of antitoxin, and 0.2 ml. of the suspension is injected into each intradermally. If the bacillus is virulent a red area of 1 to 2 cm. in diameter develops in the unprotected animal in 24 hours. The colour fades in 3 to 4 days leaving a necrotic area with a scab. The protected animal shows no lesion. By this method several cultures may be tested using the two guinea-pigs.

Since practically all diphtheria bacilli of the Gravis and Intermedius varieties are toxigenic, it usually suffices to determine that the organism is a diphtheria bacillus (by morphology, fermentation of glucose and failure to ferment saccharose) and that it belongs to either the Gravis variety (by colony form on McLeod's medium, growth in broth, and fermentation of starch) or to the Intermedius variety (by colony form and growth in broth). Only in exceptional cases is a virulence test of either variety essential.

Diphtheria is most commonly spread by droplets from the mouths either of those with the disease or of carriers. The problem of the carrier is of great importance in the spread of diphtheria. Although in the majority of cases the bacilli disappear within a few weeks after the separation of the false membrane, in the remainder they may persist for a much longer time; in a few cases for 6 months or so. Many persons who have never suffered from diphtheria are carriers of the organism. The carrier state is sometimes very intractable and may resist all the usual antiseptic measures. The most successful treatment is the removal of unhealthy tonsils and adenoids, the surgical correction of any abnormalities in the nose and the local application of penicillin.

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When there is any doubt as to whether the organism reported as *C. diphtheria* on microscopical evidence is a virulent bacillus, or when the exclusion of a suspected carrier from school or employment is involved, something more than a microscopical identification is required. ✓ The final proof that an organism is a diphtheria bacillus depends on its power of toxin production. One fairly frequently meets with bacilli which microscopically and culturally are *C. diphtheria*, but which are avirulent and atoxic. Since it has never been established that a non-toxigenic *C. diphtheria* can be transformed into a toxin-producing bacillus, and since it is well known that a toxigenic strain retains its power to produce toxin even in artificial culture for many years, one is not justified in segregating as a carrier a person in whose throat or nose non-toxigenic bacilli resembling *C. diphtheria* are found. In the case of an epidemic, where a number of healthy persons are being examined in order to find carriers, those whose throats contain what may be called "morphological" diphtheria bacilli should be isolated for the few days necessary to establish whether or not their organisms are virulent, but only those with virulent organisms should be isolated until their throats are free from them. ✓ In order to prove that an organism is a virulent *C. diphtheria* it must first be isolated in pure culture. This is most easily accomplished by the use of one of the tellurite media. The bacillus isolated fail to ferment. The best method is to inoculate a young culture on serum (one slope), suspended in broth, ✓ subcutaneously into each of two guinea-pigs, one of which has been protected by 250 units of antitoxin, injected the previous day. ✓ If the unprotected animal dies within 5 days, exhibiting the usual signs, and the other survives, the organism may be regarded as a true diphtheria bacillus. ✓ The passively immunised animal is required as certain corynebacteria which are not pathogenic for man but highly virulent for guinea-pigs may occasionally be found in the human throat and

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by T.A.F., 1 ml. of this being injected 4 weeks after the injection of A.P.T. and a further 1 ml. after another interval of 4 weeks.

Severe reactions are liable to interfere with the popularity of immunisation against diphtheria. It is, therefore, good policy to use T.A.F. as the prophylactic of choice for older children and adults. Neither A.P.T. nor F.T. should be used in the case of a person who gives a severe reaction to either of these in such persons, it

to be severe. The majority of pseudo reactors are immune, but if immunisation is considered necessary, T.A.F. should be used.

Immunisation against diphtheria should be commenced in the second half of the first year of life. If this is done, it is quite unnecessary to perform the Schick test as practically all infants of this age are Schick positive. Since satisfactory prophylactics, used as described above, convert the Schick reaction from positive to negative in the case of over 90 per cent of persons, it is not generally necessary to carry out Schick tests after immunisation although this may be done from time to time in a sample of those treated as a control of the efficacy of the prophylactics used.

When groups of older persons, such as medical students or nurses, whose occupation exposes them to a special risk of contracting diphtheria, are to be immunised, Schick testing should precede immunisation. Those giving a positive Schick reaction should receive a complete course of prophylactic. It is probably wise to administer one injection of the prophylactic (preferably T.A.F.) to those giving a negative result because, as has already been pointed out, the amount of circulating antitoxin giving a negative Schick reaction may be insufficient to protect against the disease.

The immunity resulting from a series of injections of diphtheria prophylactic is usually adequate to prevent the disease being contracted for some years. In the absence of natural exposure to the organism, however, the immunity gradually wanes. Schick reaction has been found to revert from negative to positive in 4 per cent. of immunised children within 2 years and in 18 per cent within 6 years. Any campaign for the eradication of diphtheria

In all cases where carriers are being looked for, nasal swabs in addition to throat swabs should be examined.

On account of the difficulty of finding and rendering harmless

may be conferred on those who are exposed to infection by the injection of 500 units of antitoxin. This immunity is of but brief duration, a few weeks at most. A much more lasting immunity may be established by active immunisation using a toxoid preparation.

✓ Three prophylactics are now commonly employed—formol toxoid (F.T.), alum precipitated toxoid (A.P.T.) and toxoid-antitoxin floccules (T.A.F.). ① Formol toxoid (anatoxine) is prepared by treating toxin with 0.2-0.4 per cent. formalin for 2 to 3 weeks at 37°. ② A.P.T. is obtained by adding alum to toxoid, the aluminium hydroxide which is precipitated adsorbing the toxoid which it carries down. The precipitate is centrifuged and washed to free it, as completely as possible, from non-specific material. ③ T.A.F. is the insoluble precipitate formed by the combination of toxoid and antitoxin when the two are present in optimal proportions. It also is purified by centrifuging and washing. When F.T. or T.A.F. are used as prophylactics, three intramuscular injections, each of 1.0 ml., should be given at intervals of 4 weeks. Since A.P.T. is a more efficient prophylactic than either F.T. or T.A.F., two injections usually give a satisfactory degree of immunity, provided the doses injected are adequate and the interval between them is sufficiently long. In the case of children under 6 years of age, two intramuscular injections of A.P.T., each of 0.5 ml., with an interval of 3 months between, should be regarded as the standard method of administration. If A.P.T. is used for older children or adults, which is not recommended, the first dose should be 0.2 ml. lest the reaction following a larger dose be severe. If this dose is well tolerated, a second dose of 0.5 ml. may be given 4 weeks later. If there is a severe reaction after the injection of 0.2 ml. of A.P.T., this prophylactic should be replaced

the Gravis or Intermedius varieties of diphtheria bacilli are more serious and less responsive to antitoxin than are those with the Mitis variety. Since all produce the same toxin, it is only possible to advise the use of antitoxin as early as possible and in large doses.

The term "diphtheroid bacillus" is rather loosely applied to any Gram positive, non-motile, non-sporing bacillus resembling more or less closely the diphtheria bacillus in microscopic appearance. As has been pointed out before, many bacilli appear to be identical with *C. diphtheriæ* in microscopic and cultural characteristics, save

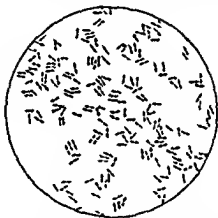


FIG 58 — *C. pseudodiphtheriticum* FROM CULTURE ON SERUM (X 800)

that they do not produce toxin. These should be called "non-toxigenic diphtheria bacilli", and the term "diphtheroid" applied only to bacilli which, while not producing toxin, may be differentiated in other ways. Some, for example, do not ferment glucose, and since all true diphtheria bacilli produce acid from this sugar, this fact is sufficient to distinguish them from the true bacilli of diphtheria.

must be planned in the light of these findings. It is suggested that every infant should receive two injections of A.P.T. during the first year of life and that one boosting dose (either of A.P.T. or T.A.F.) should be administered during the second or third and another during the fifth year.

Every case which is clinically suspicious of diphtheria should at once receive antitoxin treatment without waiting for the bacteriologist's report: 5,000 to 50,000 units, according to the severity of the case, should be administered, and this may be repeated until there is definite improvement. Cases of sore throat which are not clinically diphtheria, but in which *C. diphtheria* is present, should also receive antitoxin. Enormous doses (up to 200,000 units) are given in severe cases by some practitioners of great experience, and it is claimed that, by such treatment, mortality is much lower, paralysis of less frequent occurrence and convalescence shorter than with the more usual doses.

In connection with the administration of antitoxic serum in diphtheria, as in other diseases, the route is a matter of considerable importance. In very severe cases and in cases seen at a late stage, the route of choice is the intravenous one as the antitoxin is introduced into the circulation without delay. In other cases the intramuscular route is to be preferred to the subcutaneous. When serum is administered intramuscularly it reaches its maximum concentration in the blood in 24 hours, whereas, by the subcutaneous route, maximum concentration in the blood is attained in 72 hours.

There can be no doubt as to the efficacy of the antitoxin treatment of diphtheria if it is applied sufficiently early, but the delay of even one day greatly increases the risk of a fatal termination of the disease. If antitoxin is not administered within the first week it will probably do little to lessen the fatality, although, in the survivors, late post-diphtheritic paralysis will be less common in those who receive serum.

The severity and fatality of diphtheria vary greatly in different areas and at different times. The most important factor is the variety of *C. diphtheria* present in a community. Infections with

CHAPTER XXIX

COLIFORM BACTERIA

The term "coliform" is a convenient one to apply to a relatively large group of bacteria, the majority of which are associated, to a greater or lesser extent, with the intestinal canal. The members

organisms are included only because they are otherwise almost indistinguishable from lactose fermenting members of it. Only a

members of a long series of bacteria which, in their characteristics, are intermediate between the two and which are mainly or occasionally intestinal-in-habitat. If we had to consider only the two, which can easily be differentiated by a considerable number of criteria, there is little doubt that we would (as does Bergey's Manual) assign them to different genera. To do this would, however, force us to decide which of the intermediate forms we were to assign to one genus and which to the other. It appears to us, therefore, that both organisms with the intermediates are best allotted to the provisional genus *Bacterium*.

Bact. pneumoniae is included in this group, despite differences in habitat and pathogenicity and the failure of some strains to ferment lactose, because it closely resembles *Bact. aerogenes* in a number of important characteristics and differs from it in but few

theria. It is shorter and thicker than this organism, has tapering ends, and has not usually a beaded or granular appearance. Metachromatic granules are absent. It stains deeply and evenly with methylene blue save for the presence of a single, central, unstained septum. More rarely two unstained bars may occur.

those of *C. diphtheria*. It does not ferment any sugar and is non-pathogenic to the guinea-pig.

The Xerosis bacillus (*C. xerose*), which is frequently found in the conjunctiva and external ear, both normal and diseased, resembles *C. diphtheria* very closely in microscopic appearance. It is not pathogenic for animals and produces acid in media containing glucose, maltose, and saccharose.

Other diphtheroid bacilli are found in the respiratory, intestinal and urinary tracts, in wounds and in other localities. None of these appears to be primarily pathogenic, but they may act as secondary invaders after a lesion has been caused by other organisms.

✓ *C. acnes* is usually classed with the diphtheroids. It is a short, thick bacillus, frequently club-shaped, which grows only under anaerobic or partial anaerobic conditions. For its culture we have found that broth to which is added 1 per cent. of glucose and 1 per cent. of oleic acid, the whole being covered with a layer of oil, is a satisfactory medium. It also grows well in semi-solid glucose agar stabs. It occurs in acne pustules and comedones either alone or together with a staphylococcus.

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aerobic All ferment a variety of carbohydrates The ability to ferment lactose is regarded by us as a fundamental characteristic of the bacteria of the group and a few non-lactose fermenting organisms are included only because they are otherwise almost indistinguishable from lactose fermenting members of it. Only a small minority liquefy gelatin

The chief organisms here considered are *Bact. coli*, *Bact. aerogenes* and *Bact. pneumoniae*. The first two form the extreme members of a long series of bacteria which, in their characteristics, are intermediate between the two and which are mainly or occasionally intestinal-in-habitat. If we had to consider only the two, which can easily be differentiated by a considerable number of criteria, there is little doubt that we would (as does Bergey's Manual) assign them to different genera. To do this would, however, force us to decide which of the intermediate forms we were to assign to one genus and which to the other. It appears to us, therefore, that both organisms with the intermediates are best allotted to the provisional genus *Bacterium*.

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Bact. coli (*Escherichia coli*)

Escherich, 1886

Bact. coli is one of the most widely distributed of all bacteria. It is found in the intestines of man and of very many animals as a harmless or, more probably, beneficial saprophyte. It is of great importance to the bacteriologist for many reasons. Inside the intestine it is usually non-injurious; elsewhere in the body it is pathogenic and is to be classed with the other pyogenic bacteria. It is almost constantly found in human and animal feces, and for this reason must be familiar to the bacteriologist who has frequently to examine such material for the presence of the specific organisms of typhoid fever, dysentery or other diseases, which more or less closely resemble it. Finally, its prevalence in excreta gives it importance as an "indicator organism" in the examination of water, milk, or other food materials. If *Bact. coli* is present in these, they have been exposed, at some period, to faecal contamination.

The average size of *Bact. coli* is from 1μ to 5μ by 0.5μ ; but no great reliance should be placed on its size, since this is very variable, depending on the strain and culture medium used. It may sometimes be so short as to appear almost coccoid, at other times filamentous forms 10μ to 15μ in length may be found, especially in the urine. It possesses 6 to 8 flagella arranged round it and is motile, but usually not very actively so; some strains are so sluggish that motility can be detected in them only with great difficulty. Each bacillus is generally free, but pairs, arranged end to end, or short chains of three or four bacilli are sometimes seen. It stains readily and is Gram negative.

Bact. coli grows on all ordinary media at temperatures ranging from 10° to 45° . At the latter temperature most other coliform bacteria do not grow. It is aerobic and facultatively anaerobic. The colonies on agar plates are of large size and are circular in shape; their colour is white, greyish, or brownish, and they are rather opaque. They are usually smooth and glistening, but a not uncommon variant produces colonies with a somewhat rough surface. It does not liquefy gelatin. In broth *Bact. coli* causes a

uniform turbidity with a slight tendency to sedimentation and pellicle formation after some days. Indole is produced in cultures in broth and in peptone water. *Bact. coli* is an active fermenter of sugars, glucosides, and alcohols, with the production of carbon dioxide and hydrogen in approximately equal proportions. In glucose broth a high degree of acidity is developed. This is permanent and so the methyl-red test is positive. The most important substances fermented are lactose, glucose, maltose, mannitol, and dulcitol. Saccharose is not fermented by typical *Bact. coli* but a variety, formerly known as *B. coli communior*, does ferment this sugar. Acid and clot are produced in milk. *Bact. coli* does not give the Voges-Proskauer reaction and fails to grow in Koser's medium. On blood agar some strains are hæmolytic, but

non-motile, saccharose-fermenting, serologically homogeneous strain known as D433, which is frequently present in the faeces in

per bounds, is interfered with, as in typhoid fever and dysentery, or the whole intestinal wall is damaged, as in strangulated ilia or volvulus, the common *Bact. coli* of the intestine can penetrate into neighbouring tissues and cause suppuration. Appendicitis, peritonitis, and peri-rectal abscesses are sometimes caused by *Bact. coli*, either alone or associated with other organisms. If the flow of bile is arrested the organism may penetrate into the gall-bladder, setting up a cholecystitis. It may infect the urinary system, particularly in the female or in the male after

pelvic or subphrenic abscesses, empyema or abscess in the lung. *Bact. coli* septicæmia is uncommon and rarely occurs save as a terminal phenomenon of diabetes and other chronic diseases; but

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local conditions, which are almost certainly due to infection by the blood stream, may be seen in the occasionally noticed pneumonia, pleurisy, osteomyelitis, and meningitis caused by the bacillus. *Bact. coli* is definitely a pyogenic organism, and even injections of dead bacilli may cause local abscesses.

When *Bact. coli* has taken on a definitely pathogenic role the patient's serum is frequently found to agglutinate the organism responsible for the condition. Normally a person's serum does not agglutinate a saprophytic, intestinal *Bact. coli*.

Most strains of *Bact. coli* are sensitive to the action of sulphonamides which, as they are excreted in the urine, are of special value



FIG 59—*Bact. coli* IN DEPOSIT OF URINE FROM CASE OF CYSTITIS (X 950)

in the treatment of infections of the urinary tract. The organism is normally highly resistant to penicillin but is sensitive to streptomycin.

For the isolation of *Bact. coli* from faeces, urine, water or other material, one of the streptococcus agar may be taken as a type, produced colonies.

sugar and other media mentioned above in order to determine, as exactly as possible, whether the organism is to be classed as a true *Bact. coli* or as one of the somewhat similar organisms.

found to be viscid or mucoid when touched with the platinum wire. On an agar slope the growth, which is luxuriant, tends to gravitate to the bottom of the tube. On blood-agar a wide zone of hæmolysis is produced around each colony. In a gelatin stab a white line of growth is formed, and on the surface the organisms

ally produced. Probably owing to the occurrence of various strains the fermentative activities of the bacillus are varied. Most

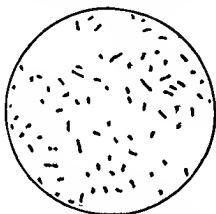


FIG 60—*Bact. pneumoniae* SHOWING CAPSULES ($\times 750$)

usually glucose, mannitol, maltose and saccharose are acted upon, acid and gas being produced; lactose may or may not be fermented. The Voges-Proskauer reaction is usually positive and the methyl-red test negative. Most strains of *Bact. pneumoniae* grow in Koser's medium.

The organism is fairly commonly found about the respiratory tract, and frequently appears to play no pathogenic rôle. It may, however, in a small percentage of cases, be the cause of pneumonia of a very severe type. It has occasionally been the cause of abscess formation in the respiratory tract, of suppuration in the nasal and frontal sinuses and in the antrum of Highmore. Cases

hydrogen. The bacillus does not liquefy gelatin; indole is not usually produced; the Voges-Proskauer reaction is positive. The bacillus is capable of growing freely in medium, such as that of Koser, in which the only source of carbon is a citrate. Some strains of *Bact. aerogenes* are encapsulated, which may give rise to difficulty in distinguishing them from *Bact. pneumoniae*.

The organism appears to be chiefly a saprophyte, being found in soil, water, and grain; it is one of the common causes of the souring of milk. It is not uncommonly found in small numbers in human feces and may occasionally be pathogenic for man, causing cystitis or other types of inflammation; it has been found in the blood stream in septicemia in a few cases.

The chief importance of this bacillus lies in the fact that it is commonly found in water supplies, particularly in tropical countries and, as it ferments lactose, it may be confused with *Bact. coli*. Since it is widely distributed in nature, its presence in water has not the significance attached to *Bact. coli*, which is predominately fecal in origin.

Fairly closely related to *Bact. aerogenes* is *Bact. cloacae* (*Aerobacter cloacae*), which, however, causes liquefaction of gelatin.

Bact. pneumoniae (*Klebsiella pneumoniae*)

Friedländer, 1882

Bact. pneumoniae is a pleomorphic organism which may vary in length from 0.6μ to 4.0μ and in thickness from 0.5μ to 1.2μ . In the body it is most commonly seen in almost coccal form, and was, in fact, originally described as a coccus and for some time was confused with the pneumococcus. It occurs singly or, very commonly, in pairs; much more rarely in short chains. It is non-motile and Gram negative. As seen in secretions or in early cultures it is supplied with a well-marked capsule. No spores are produced.

It grows readily on ordinary media, best under aerobic conditions, and either at the temperature of the body or at 20° . On agar it produces large, grey, or white, raised colonies, which appear moist or semi-fluid and tend to be confluent. These are

found to be viscid or mucoid when touched with the platinum wire. On an agar slope the growth, which is luxuriant, tends to gravitate to the bottom of the tube. On blood-agar a wide zone of hæmolysis is produced around each colony. In a gelatin stab

ment of a slimy sediment and of a pellicle. Indole is only occasionally produced. Probably owing to the occurrence of various strains the fermentative activities of the bacillus are varied. Most

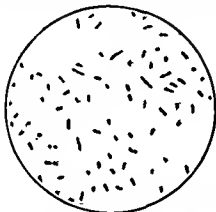


FIG. 60 —*Bact. pneumoniae* SHOWING CAPSULES ($\times 750$)

usually glucose, mannitol, maltose and saccharose are acted upon, acid and gas being produced; lactose may or may not be fermented. The Voges-Proskauer reaction is usually positive and the methyl-red test negative. Most strains of *Bact. pneumoniae* grow in Koser's medium.

The organism is fairly commonly found about the respiratory

of otitis media, empyema, pericarditis, meningitis and septicæmia have been produced by it, and it is commonly associated with both acute and chronic bronchitis.

The majority of strains of *Bact. pneumonia* fall into one of three serological types but there are probably further types not yet differentiated. As with the pneumococci, type specificity depends on the presence of a carbohydrate substance in the capsule.

Bact. pneumonia is resistant to penicillin but is usually sensitive to sulphonamides and streptomycin.

A bacterium either identical with or closely related to *Bact. pneumonia* is found both inside and outside the cells of Mikulicz in the rare disease, rhinoscleroma. In ozæna, a similar bacillus is almost constantly found and is believed by some to be causative of the condition.

CHAPTER XXX

SALMONELLAE

This genus, named after the American bacteriologist Salmon, comprises a large number of species which share the same general biochemical characteristics and tend to produce the same disease in the animal body. The nomenclature of this genus has undergone several changes. Some of the species earlier described were named after the disease with which they were associated and these names, such as *Salm. cholerae*, *Salm. typhi*, *Salm. paratyphi* and *Salm. enteritidis*, have, for the most part, been retained. A few species have been called after the persons who discovered or worked with them (e.g. *Salm. schottmuelleri*) or of the patient from whose body they were isolated (e.g. *Salm. thompsoni*). In recent years, however, it has become the accepted practice to give to a new species the name of the locality where it was first isolated. Hence we have *Salm. dublin*, *Salm. london*, *Salm. poona* and so on.

This system of nomenclature. This might be achieved by retaining specific rank only for commonly isolated species, the names of which have become established by tradition, the remaining species being grouped together under a few names and distinguished, when necessary, by an appended antigenic formula.

From the point of view of the medical bacteriologist, the most important species of this genus are those responsible for enteric fever in man.

its diagnosis to the next chapter.

All salmonellæ are Gram negative, non-sporing rods, morphologically indistinguishable from *Bact. coli*. They possess flagella and are usually actively motile but, when freshly isolated from the body, motility may be sluggish or even absent. One or more subcultures in broth, however, are usually sufficient to restore active motility. Salmonellæ are not, as a rule, capsulated, although some species, notably *Salm. paratyphi B*, occasionally give rise to mucoid colonies in which the individual organisms are surrounded by capsular material.

Growth occurs readily on unenriched nutrient agar over a wide

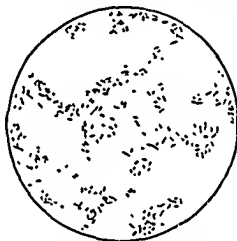


FIG 61.—*Salm. typhi* FROM AGAR CULTURE.

temperature range, although optimally at 37°. Growth under anaerobic conditions is poor. Colonies of freshly isolated strains are almost invariably circular in outline with a smooth, glossy surface, and may be difficult to distinguish from those of *Bact. coli*. They are, however, more translucent and have a more delicate texture. The colonies formed by strains which have frequently been subcultured on artificial media tend to be rough, irregular in surface and irregular edge. This variation, which and which is usually called a loss of virulence. A few *paratyphi A*, grow less pro-

fusely and give rise to colonies which are appreciably smaller than is normal for the genus. On desoxycholate-citrate medium, which

desoxycholate by the air
organism on this medium

Salm. paratyphi B from faeces, the selective medium of choice is that of Wilson and Blair on which both species produce black colonies surrounded, in the case of *Salm. typhi*, by a gun-metal sheen. This medium may, however, partially or completely suppress the growth of other *Salmonella* species and should, therefore, always be used in conjunction with desoxycholate-citrate agar. On MacConkey's medium, colonies are rather translucent and almost colourless.

Of the liquid enrichment media used to facilitate isolation of salmonellæ from faeces, tetrathionate and selenite are the two most satisfactory.

Salmonellæ cause a uniform turbidity in broth. They do not liquefy gelatin. Indole is never produced.

The fermentative activities of the genus are more restricted than

have been described.

The principal pro
the various species
these may undergo,
Chapter XVIII.

As regards flagella, only about one fifth of the species are monophasic, the remainder displaying diphasic variation. The combination of O antigens which characterises the surface of the bacteria

not found in the species of other groups. By reference to the table on page 236, which shows the antigenic formulæ of a number of representative species, it will be seen that antigens IV and V characterise the species of Group B, antigen VI those of Group C and antigen IX those of Group D. It should be noted that antigen XII, for example, is common to Groups A, B and D so that an antiserum prepared against, say, *Salm. typhi* will agglutinate not only species of the same group, such as *Salm. enteritidis*, but also *Salm. paratyphi A*, *Salm. paratyphi B* and *Salm. typhimurium* which belong to other groups. Antisera specific for each group are, however, easily prepared by absorption of agglutinin and are of considerable value in identification. If, for example, *Salm. typhi* O antiserum, containing antibodies against antigens IX and XII, is absorbed with a suspension of *Salm. paratyphi B*, containing antigens I, IV, V and XII, all antibodies except those directed against antigen IX will be removed and the antiserum thereby rendered specific for species of Group D.

In view of the large number of *Salmonella* species and the complexity of their antigens, the adoption of a logical system for their identification is essential. When an organism has been isolated, the morphological, cultural and biochemical characteristics of which conform to those of the genus, the first step in its antigenic analysis is to test the growth from culture on solid medium against specific O group antisera, using the slide agglutination technique. If, for example, agglutination occurs with Group B antiserum only, then subsequent investigation may be limited to the H antigens of the comparatively small number of species belonging to this group. A broth culture of the organism is, therefore, tested by Dreyer's method for agglutination by H antisera specific for the phase 1 antigens of each of these species, of which *Salm. paratyphi B* and *Salm. typhimurium* are those most commonly isolated from man. If no agglutination occurs, it is probable that the organism belongs to a diphasic species and that its flagella are

in phase 2. It should then be plated out and colonies picked and tested against phase 2 antiserum. A broth culture of an inagglutinable colony, which may be presumed to be in phase 1, is finally tested against the phase 1 antisera previously employed. Knowledge of the O group to which a *Salmonella* belongs and of its H (phase 1) antigens is usually sufficient to determine its species. In the case of some rare species, however, the antigens of phase 2 must also be identified by the use of absorbed, antigen-specific sera in order to determine the species with certainty. Such an investigation would be necessary to distinguish, for example, between *Salm. paratyphi B* and *Salm. abony*.

In the case of freshly isolated strains of *Salm. typhi*, no agglutination may be obtained with homologous O antiserum owing to the presence of Vi antigen. This antigen, discovered by Felix, is present in all freshly isolated strains of *Salm. typhi* and is associated with the virulence of the species. It lies superficially in the bacterial bodies and, by covering or masking the O antigens, protects those organisms possessing it from the action of O antiserum. By immunisation of an animal with killed suspensions of Vi-containing *Salm. typhi*, Vi antibodies are produced, though their titre in the serum is usually considerably lower than that developed against the H or O antigens. By absorption of the antiserum with a suspension of a strain of *Salm. typhi* which has lost its Vi antigen completely, while retaining its H and O antigens, a pure Vi antiserum is obtained. This serum not only causes agglutination of suspensions of Vi-containing organisms but also protects mice against many M.L.D. of virulent *Salm. typhi*, whereas O antiserum does not.

While O antigens are unaffected by heat and by treatment with acids, alkalis and phenol, Vi antigen is destroyed by these agents, thereby unmasking the O antigens and rendering the organisms agglutinable by O antiserum. For the production of therapeutic Vi antisera, or of vaccines containing Vi antigen for prophylactic use, the method used to kill the organisms is of importance. If, for example, they are killed by heat and preserved in 0.5 per cent. phenol, as is usual with the majority of vaccines, all the immuno-

genic properties of Vi antigen are lost. For this reason, Felix recommended the use of typhoid vaccines the organisms of which had been killed by 75 per cent. alcohol and were suspended in 23 per cent. alcohol, since such treatment preserves to the full the ability of Vi antigen to stimulate the production of protective antibodies.

Loss demonstrates that only those which have suffered a partial loss of the antigen are agglutinated by both Vi and O antisera, when loss of the antigen is complete, agglutination occurs with O antiserum only.

The reason why Vi antigen confers virulence on *Salm. typhi* is a matter both of interest and of importance. That the antigen is neither itself toxic nor directly responsible for virulence, is shown by the fact that a few rare *Salmonella* species of low virulence, as well as certain strains of *Bact. coli* of no higher pathogenicity than is usual for this organism, possess a surface antigen serologically indistinguishable from the Vi antigen of *Salm. typhi*. Vi antisera prepared by immunisation of animals with these strains protect mice against virulent *Salm. typhi*. It is probable that the ability of *Salm. typhi* to produce typhoid fever is a property of its O antigens, the role of Vi being the passive one of protecting the organisms from the opsonic and killing action of O antibodies. It has been shown experimentally that suspensions of *Salm typhi*

has been made possible by Craigie's discovery of a bacteriophage which, while acting only on the Vi antigen of *Salm. typhi*, has the property of acquiring, by adaptation, a high degree of specificity for certain strains on which it is propagated and, thereby, of distinguishing them from other strains possessing a serologically identical Vi antigen. All those strains which show specific lysis by this bacteriophage after it has been adapted to one of them, are

said to belong to the same type. Bacteriophage typing of *Salm. typhi* has proved of considerable value in tracing the particular carrier responsible for an outbreak of typhoid fever.

In addition to the species mentioned above, *Vi* antigen is found also in *Salm. paratyphi C*. We believe that the term "*Vi*" should be reserved

antigens se
species. Fe

surface antigens in *Salm. paratyphi A*, *Salm. paratyphi B* and *Salm. typhimurium*. These he also describes as *Vi* antigens, though they differ serologically from that of *Salm. typhi*, as well as from one another, and never completely mask the *O* antigens underlying them.

Exotoxins are not produced by *Salm. typhi* or by any other species in the genus *Salmonella*. *O* antigens, however, are powerful endotoxins and, since they are soluble, can exercise their effect without death and dissolution of the organisms containing them. Chemical analysis has shown that the *O* complexes of *Salm. typhi* and *Salm. typhimurium*, which have been investigated more fully than those of other species, are composed principally of polysaccharide hapten, which confers specificity on the antigen.

Increasing knowledge of the genus in recent years has done much to alter our views on the infectivity of the various *Salmonella* species for animals and man. While it may be asserted that

animals. Conversely, every species formerly thought to cause disease only in animals or birds has now been isolated from human infections. Host adaptation by passage probably plays an

genic properties of Vi antigen are lost. For this reason, Felix recommended the use of typhoid vaccines the organisms of which had been killed by 75 per cent. alcohol and were suspended in 23 per cent. alcohol, since such treatment preserves to the full the ability of Vi antigen to stimulate the production of protective antibodies.

Loss of Vi antigen occurs readily in artificial culture and can be demonstrated by the use of pure Vi and O antisera. Organisms possessing an intact Vi antigen are agglutinable by Vi antiserum only; those which have suffered a partial loss of the antigen are agglutinated by both Vi and O antisera; when loss of the antigen is complete, agglutination occurs with O antiserum only.

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The sub-division of *Salm. typhi* into a number of different types has been made possible by Craigie's discovery of a bacteriophage which, while acting only on the Vi antigen of *Salm. typhi*, has the property of acquiring, by adaptation, a high degree of specificity for certain strains on which it is propagated and, thereby, of distinguishing them from other strains possessing a serologically identical Vi antigen. All those strains which show specific lysis by this bacteriophage after it has been adapted to one of them, are

but it may be due to any species except *Salm. typhi*. The fatality rate is probably from 5 to 10 per cent.

➤ In the third type of disease, *Salmonella gastro-enteritis*, only the alimentary tract is involved and general invasion of the body does not occur. This is a variety of food poisoning and may be of two kinds, infective or toxic. In the infective type, the organisms are ingested in contaminated food, usually meat. There is commonly an incubation the time necessary for intestine. In addition to severe and prolonged over several days, there is usually pyrexia. The organism can frequently be isolated from the stools and vomit as well as from the infected food. The consumption of eggs of ducks infected with *Salm enteritidis* or other species has been responsible for many outbreaks of food poisoning of this type, the organisms apparently being transmitted to the egg yolk prior to laying. The toxic type is usually due to the consumption of re-cooked food, usually meat. The food is infected indirectly by flies or hands, or directly by the faeces of rodents, during storage after the first cooking. The organisms, growing in the food, are supposed to produce an irritant substance or toxin. The organisms are killed on re-cooking so that the disease is due solely to the action of this substance. Although this theory fits the facts, it must be admitted that a heat-stable toxin of the kind postulated has not yet been demonstrated experimentally. The onset of symptoms occurs rapidly, usually within an hour of eating the food. Diarrhoea and vomiting may be severe but are of much shorter duration than in the infective variety. There is usually no pyrexia and the causative organism cannot be isolated from either patient or food. Unlike enteric fever or *Salmonella septicæmia*, the fatality rate in both varieties of gastro-enteritis is low. The condition may

important part in determining pathogenicity for a given animal species. For example, human epidemics of *Salm. paratyphi B* infection have been described in which the early cases suffered only from gastro-enteritis, while cases of typical enteric fever developed later in the epidemic.

The salmonellae are strict pathogens and have no habitat other than the human or animal body. The source of human infection is, therefore, a human or animal case or carrier, the organisms being excreted in the faeces or urine and transmitted by food or water which is ingested by another individual. The portal of entry of the organisms is always the mouth, irrespective of the subsequent sequence of events. Any organism with the even-

enteritis, of which the most important is enteric fever.

The term "enteric fever" should be used to indicate the clinical type of disease about to be described, irrespective of the causative *Salmonella* species. The terms "typhoid fever" and "paratyphoid fever", often used synonymously with "enteric fever", imply a bacteriological diagnosis (of infection with *Salm. typhi* or *Salm. paratyphi A, B* or *C* respectively) and should be used only when this has been established. Enteric fever is characterised by fever of continuous type, enlargement of the spleen, usually a "rose-spot" rash and leucopenia. Headache and constipation are common in the early stages of the disease but diarrhoea with "peppery" stools is usual later when ulceration of the bowel is present. The diagnosis is confirmed by blood culture, and later from the faecal excretion. The mortality rate of enteric

The next clinical type of infection to be considered is *Salmonella* septicaemia. This resembles a septicæmia due to the pyogenic cocci, with a spiky temperature of intermittent or remittent type. The disease may run an uncomplicated course of from 10 days to several weeks or, after a varying period of simple pyrexia, localisation with pus formation in the urinary system, serous cavities

CHAPTER XXXI

✓ ENTERIC FEVER

Salmonella typhi (*Salmonella typhosa*, *Eberthella typhosa*)
Eberth and Gaffky, 1884

Salmonella paratyphi A
Brion and Kayser, 1902

Salmonella paratyphi B (*Salmonella schottmuelleri*)
Schottmüller, 1900

Salmonella paratyphi C (*Salmonella hirschfeldii*)
Hirschfeld, 1919

The mode of spread of salmonellæ in general has been dealt with in the previous chapter. In view of the importance of enteric fever, and of the influence which a proper understanding of the principles underlying its diagnosis and control may have on public health, it is necessary to consider this disease in greater detail.

As has already been described, enteric fever is most commonly spread throughout the world by *Salm. typhi*. Other *Salm.*

while *Salm. paratyphi A* or *C* is seldom, if ever, isolated. In India and in South America, on the other hand, *Salm. paratyphi A* is a common cause of enteric fever and *Salm. paratyphi B* a very rare one. During the last war, the isolation of a variety of *Salm. enteritidis* from cases of enteric fever in India was by no means uncommon although invasive disease due to this species is extremely rare in Europe. In British Guiana, *Salm. paratyphi C* is the dominant species.

There is no doubt that *Salm. typhi* is an exclusively human

be caused by any *Salmonella* species, with the exception of *Salm. typhi*. *Salm. typhimurium* and *Salm. enteritidis*, which are natural pathogens of mice and rats respectively, are most commonly incriminated.

While there is little doubt that animals are a very important source of infection in food poisoning due to salmonellæ, a considerable volume of evidence suggests that human carriers, who are food-handlers, may be responsible for a considerable proportion of outbreaks. There is no doubt that both healthy and convalescent human carriers of those salmonellæ most frequently responsible for gastro-enteritis do exist, but proof of their role in the causation of an outbreak is often very difficult to obtain.

The laboratory animal most widely used in the study of *Salmonella* pathogenicity is the mouse. In this animal, *Salm. typhimurium* causes a natural infection resembling enteric fever in man. Use has been made of this host-organism relationship to study the epidemiology of enteric fever under laboratory conditions.

✓ Although *Salm. typhimurium* is more pathogenic for the mouse than is any other *Salmonella* species, including *Salm. typhi*, intraperitoneal inoculation of virulent strains of the majority of other species produces a rapidly fatal septicæmia, the M.L.D. of *Salm. typhi* being between 50 and 100 million organisms. The mouse is the most convenient animal to employ for testing the efficacy of vaccines used for the prophylaxis of enteric infections in man.

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There is no doubt that *Salm. typhi* is an exclusively human

pathogen and that, although infections of lower animals with *Salm. paratyphi A* and *B* have been reported, these two species, together with *Salm. paratyphi C*, are predominantly human pathogens. When enteric fever is caused by species which normally cause disease of animals and birds and which, in man, usually produce only a mild illness, the evidence to suggest that the organism is the cause of the disease for man must be based on the fact that the organism is the aetiology of enteric fever in general, therefore, we will assume that the ultimate source of infection is the human case or carrier who excretes the organism either in the faeces or, less commonly, in the urine. In order that the disease may be spread, the organisms must be conveyed thence in sufficient numbers to the alimentary tracts of other people. Among communities served by primitive or faulty systems of sanitation, spread is most frequently effected by water from rivers or shallow wells to which infected excreta have gained access. The introduction of good methods of sewage disposal and the purification of water supplies have enormously reduced the prevalence of enteric fever in civilised countries where the disease is now transmitted chiefly by food which has been handled by carriers. Milk and ice-cream are not uncommonly the vehicles of spread. A knowledge of those persons who, after infection, become chronic carriers and their exclusion from employment involving the handling of food are, therefore, of fundamental importance in the control of the disease.

A not uncommon mode of infection is the consumption of shell-fish taken from the estuary of a river into which is discharged the sewage of a city or town. In countries having a hot, dry climate and where excreta may be exposed to the air, salmonellae are often

the gall-bladder and the lymph follicles of the small intestine are typically involved. The sequence of events following ingestion of *Salm. typhi* has been traced by examination of material taken from various parts of the body throughout the course of the disease of in which

disease and after death. Corroborative findings have been obtained indirectly by experiments on mice infected by feeding with cultures of *Salm. typhimurium* which produces in these animals a disease similar to enteric fever in man.

It is probable that the bacteria which have entered the body by the mouth and have survived the acidity of the gastric juice do not

are rapidly removed by the reticulo-endothelial cells and especially by those of the spleen, liver and bone-marrow which form the principal foci of infection. In them, and in the mesenteric lymph glands, the organisms multiply and produce a secondary bacteræmia which is of greater severity than the first and which marks the onset of the actual disease. At this stage, the bacteria are

through two channels. The first of these is the infected Peyer's patches and solitary lymph follicles of the small intestine which

gall-bladder which is constantly infected. This explains the finding of the organisms in almost pure culture in the duodenal fluid. After recovery from great importance

In about 25 per cent of cases of typhoid fever, *Salm. typhi* may be found in the urine after the third week. Since the urine is free from pus, it is probable that there is no actual infection of the

urinary tract. In contrast to this, ^Bwhen localisation of infection in the renal system occurs in *Salmonella* septicæmia, the condition is always a purulent one.

✓ Bone abscesses occasionally occur as a sequel to typhoid fever and in them *Salm. typhi* may persist for long periods after convalescence.

(2) Following an attack of typhoid fever, the great majority of cases cease to excrete *Salm. typhi* during convalescence. About 5 per cent. of cases continue to excrete the organism during 6 months after the attack, while some 3 per cent. are still excretors at the end of a year. ✓ The majority, if not all, of those who are still excreting typhoid bacilli a year after convalescence become permanent carriers and continue to excrete the organism indefinitely. Such carriers are largely responsible for outbreaks of the disease. Less information is available concerning the duration of the carrier state in enteric fever due to other *Salmonella* species, but permanent carriers of *Salm. paratyphi B* are known to occur and their incidence in relation to infected persons is probably of the same order as is found in typhoid fever.

carriers is very much higher among women than among men. The seat of residual infection in faecal carriers is the gall-bladder and there is a convincing amount of evidence to show that the higher carrier rate in women is associated with their greater proneness than men to gall-bladder disease. Women who suffer an attack of cholecystitis during the course of typhoid fever are much more likely to become permanent carriers than are those without gall-bladder symptoms.

Strictly speaking, the term "carrier" connotes any person who harbours an organism causative of infectious disease. It has been suggested that, following an attack of typhoid fever, *Salm. typhi* may remain in the body without being excreted but, with the exception of cases having a bone infection as a sequel to their

attack, carriers of this sort must be very rare and do not constitute a source of infection. For practical purposes, therefore, a carrier may always be regarded as meaning also an excretor. Although permanent carriers form the main reservoir of infection, it should not be forgotten that, in areas where enteric fever is endemic, or during an epidemic, there are always a number of immune persons who harbour and excrete the causative organisms for a short time although they neither have had nor subsequently develop the disease. Others, having a substantial but lesser degree of immunity, may suffer such a mild attack that they remain ambulant and the true nature of their illness passes unrecognised.

Methods used for the prevention of enteric fever fall into two categories. The first and more important aims at breaking the chain which links the chronic carrier to the fresh case, by general public health measures such as the improvement of sanitation, purification of water supplies and control of carriers. The second method, introduced by Almroth Wright at the end of the last

T.A.B. vaccine has been subjected to extensive trials under field conditions during two World Wars, when conditions have been especially favourable for the spread of enteric fever, and there can be little doubt that its use has resulted in a marked lowering in the incidence of the disease among populations exposed to the risk of infection. Experience gained during World War II, however, strongly suggests that the severity and fatality rate of the

ably, have not been long isolated. Until recently, only heat-killed, phenolised vaccines were employed. On account of destruction of Vi antigen by both heat and phenol, Felix advocated the use of alcoholised T.A.B.-vaccine, but considerable experience of its

use in the British Army during the past few years suggests that, in spite of its theoretical superiority, it is no better as a prophylactic than is the older type of vaccine.

Heat-killed, phenolised T.A.B. vaccine usually contains, in each millilitre, 1000 million *Salm. typhi* and 750 million each of the paratyphoid organisms. Two doses of 0.5 and 1.0 ml. are given at an interval of 2 to 3 weeks. A red, painful swelling frequently develops at the site of injection within a few hours and may be followed by general constitutional disturbance, with fever and malaise. Such symptoms rarely persist for longer than 48 hours. The recommended dosage of alcoholised vaccine is one-half that of heat-killed, phenolised vaccine and both local and general reactions following its administration are usually slight. For those persons who continue to be exposed to the risk of infection, a single annual "boosting dose" of vaccine is recommended.

There is as yet no really effective specific treatment for enteric fever. Felix advocated the use of *Salm. typhi* antiserum, containing both Vi and O antibodies but, although some favourable results have been reported, this form of treatment has not been widely adopted. *Salm. typhi* is moderately susceptible to the action of penicillin, its sensitivity being so increased by the presence of small amounts of sulphathiazole as to fall within the effective therapeutic range of large doses of penicillin. Reports as to the efficacy of the treatment of typhoid fever with penicillin and sulphathiazole are conflicting. Chloromycetin is stated to have given very good results in the treatment of the disease. Promising results have been obtained by the treatment, with sulphathiazole and massive doses of penicillin, of a small, but well controlled, series of permanent typhoid carriers.

The Laboratory Diagnosis of Enteric Fever

A bacteriological diagnosis of *Salmonella* infection can be made with certainty only by isolation and identification of the causative organism. Even in a clinically typical case of enteric fever, the establishment of a bacteriological diagnosis is of importance for, although the majority of such cases are due to *Salm. typhi*, the

isolation of some other species may suggest not only an alternative epidemiological approach, but also a different prognosis.

1. Blood Culture *During the 1st week*

In enteric fever, isolation by blood culture is the method of choice. The earlier it is performed the greater is the chance of success. During the first week, *Salmonella typhi* can be isolated from the blood of 90 per cent. of cases of typhoid fever. The percentage of successful isolations falls to rather more than seventy in the second week, to sixty in the third week and to forty in the fourth week. In the event of early failure, it is clearly worth while continuing blood culture throughout the course of the disease.

It is desirable to use glucose broth in addition to broth containing 0.5 per cent. of bile salts when performing blood culture. The

contaminating organisms, by preventing clotting of the blood and, what is most important, by inhibiting the bactericidal properties of the patient's serum and leucocytes. The addition to this medium of 1 in 15,000 gentian violet assists in completely suppressing the growth of skin staphylococci and other contaminating organisms without affecting the multiplication of salmonellae. About 5 to 10 ml. of blood, taken from a vein in the ante-cubital fossa with a sterile syringe, should be added to 100 or 150 ml. broth in a special bottle. After overnight incubation, a loopful is spread on the surface of a solid medium from which a pure culture can be obtained for identification. Since the growth of small numbers of salmonellae in blood culture may be delayed, the culture should be plated in this way at daily or 2-day intervals for a week before being reported negative.

2. Faecal Culture

Turning now to the frequency of successful faecal culture at different stages of the disease, it has been found that during the

first week of typhoid fever *Salm. typhi* can be isolated from the stools in rather more than 50 per cent. of cases. This figure falls somewhat during the second week and then rises to a peak figure of nearly 80 per cent. towards the end of the third week. Thereafter, the chances of successful isolation fall off rather sharply. These figures apply only to the average case and it is clear that the chances of isolation from any individual case will be increased if examination of faeces is carried out at intervals from the onset of symptoms.

Cultures of faeces should be made by inoculating a tube of tetrathionate broth or one of selenite broth, and by plating on both

medium
broth

all three plates are examined and suspicious colonies plated on MacConkey's agar from which, after overnight incubation, non-lactose-fermenting colonies are picked for further examination. The number of positive isolations which follow the use of an enrichment medium and two different selective media, in the manner described, is so much higher than when only one selective medium is used that the additional trouble involved is warranted.

3. Culture from the Urine

After the third week, the urine should be cultured at intervals not only with the object of making a bacteriological diagnosis when isolation from other sources has failed, but also to identify those cases who may later become urinary carriers.

A fresh specimen of urine should be obtained under aseptic conditions and several loopfuls spread over the surface of MacConkey's medium from which colonies can be picked for further examination. Only in the case of specimens of urine which have become heavily contaminated with other organisms need a highly elective medium be used for isolation.

4. Culture from Other Sources

Occasionally, in typhoid fever, *Salm. typhi* may be isolated from the rose spots, sputum and vomit. In the post-mortem examina-

tion of persons suspected of having died from enteric fever, cultures should be made from the spleen, mesenteric glands, bone-marrow and gall bladder in all of which the causative organism is constantly present. Since pure cultures can usually be obtained from all these sources, direct plating of the material on Mac-Conkey's medium is sufficient for isolation.



5. The Widal Test

This test is based on the fact that

in tubes against suspensions of those *Salmonella*-species most commonly responsible for enteric fever, that species against which the serum shows the highest titre may be presumed to be the cause of the infection. The test is rendered more complex by the fact that, since salmonellæ possesses both H and O antigens, it is

motile strains with formalin which fixes the flagella in such a way as partially to mask the body antigens. Suspensions which are agglutinated by O, but not by H, agglutinins are obtained either by the use of permanently non-motile strains or by treating suspensions of motile organisms with alcohol which destroys flagellar agglutinability.

In the case of sera from persons who have not previously

when H suspensions are used, partly because of the presence in the serum of residual H agglutinins which may persist for years and also because there is a tendency for the titre of such agglutinins to increase in response to the non-specific stimulus of a quite unrelated infection—the anamnestic response. In contrast to this, the titre of O agglutinins reaches a lower level following injection

of T.A.B. vaccine, falls much more rapidly than does the H titre and is not subject to an anamnestic rise. These advantages of the use of O suspensions in the Widal test are offset by two disadvantages. Firstly, as we have seen, O antigens are much less species specific than are H phase 1 antigens, so that infection with, say, *Salm. paratyphi B* often produces as high an O titre against *Salm. typhi* as against the homologous species. Secondly, when the sera of uninoculated, healthy individuals are tested by Felix's technique against O suspensions of *Salm. typhi*, a number are found to show agglutinin titres of up to 1 : 640, so that lower titres are of little value as evidence of infection.

3. Finally, it should be noted that an appreciable proportion of cases of typhoid fever fail to develop H agglutinins at all, while, in others, the titre of O agglutinins may not rise significantly above their normal level. If the serum of a patient is tested on several occasions during the course of the disease, an increase of more than 100 per cent. in either the H or O titre may be taken as evidence suggestive of infection.

may show agglutination of Vi-containing suspensions at a dilution of up to 1 : 20, great caution must be exercised in assessing the significance of results.

✓ of considerable diagnostic value in persons who have not previously been immunised with T.A.B. vaccine.

✓(b) In immunised persons, H agglutination is valueless. In them, the occurrence of an O titre of 1 : 1280 or over is very suggestive of infection but, owing to the relative non-specificity of O antigens, little can be inferred as to the identity of the infecting species.

✓(c) A standard Vi titre of 1 : 40 or over strongly suggests infection with *Salm. typhi*.

✓ (d) A progressive rise in either H or O titre is suggestive of infection but is by no means diagnostic.

✓ (e) The absence of an H, O or Vi titre in a patient's serum never excludes the possibility of *Salmonella* infection.

We see, therefore, that the Widal test is rarely able to confirm, and can never exclude, a diagnosis of enteric fever, especially in immunised persons. Under no circumstances should the test replace bacteriological examination of material from suspected cases.

Dreyer's technique should be used for H type agglutination and Felix's technique for the detection of O and Vi agglutinins. The antigens used in the test depend on the local prevalence of the various *Salmonella* species. In the British Isles, H and O suspensions of *Salm. typhi* and *Salm. paratyphi B* (phase 1), as well as a suspension of a Vi-R variant of *Salm. typhi* for the detection of Vi agglutinins and of *Salm. typhimurium H* (phase 2), are those most commonly employed for routine work.

6. The Identification of Carriers

The main difficulty in the identification of faecal carriers lies in the intermittency with which the organism is excreted. Many consecutive stool examinations may prove negative before the organism is finally isolated, when it may be found in considerable numbers. Thus, although one positive stool examination will prove a person to be a carrier, it is almost impossible to set a limit to the number of specimens which should be examined with negative results in order to exclude the carrier state. All we can say is that if a person having repeatedly negative stools is really a carrier, then he is much less a source of danger to the community than is a regular and prolific excreter. Since the seat of infection in the permanent faecal carrier is the gall-bladder, the chances of isolation are considerably increased if a flow of bile is promoted by a dose of calomel, followed by a saline purge. The first stool following this treatment is discarded, the second and third stools being pooled and used for examination. It is important that the stool be fluid so that the organisms in it are evenly

distributed and, therefore, *not so likely* to be absent from the random samples selected for investigation. When delay between passage of the stool and its examination is unavoidable, the viability of salmonellæ may be preserved by mixing the fæces with buffered glycerol saline (see Chapter XLVIII). Examination is carried out as described above. An alternative method for the identification of fæcal carriers is examination of the bile. Samples of bile are collected by means of a duodenal tube and loopfuls plated on MacConkey's medium. A highly selective medium is not required since the bile is unlikely to be contaminated by coliform organisms.

For the identification of urinary carriers, samples of urine may be tested by plating on MacConkey's medium as has already been described. A more sensitive method is to plate out several loopfuls of the urinary deposit after centrifugation of the urine. Since urinary carriers may be intermittent excretors, at least six samples of urine, passed on different days, should be examined before the carrier state can be excluded with any degree of confidence.

Unlike *H. typhi*, which is said to disappear after infection, *typhi* has been eliminated from the system. It is not certain that the presence in the serum of Vi agglutinins having a standard titre of 1:5 or over indicates persistence of infection following an attack of typhoid fever. Not all workers agree with the validity of this view. The consensus of opinion to-day is that the test is a useful one for "screening" carriers, that is for selecting from a large body of possible carriers those who are probable carriers and so reducing the number of persons whose excreta must be examined.

CHAPTER XXXII

BACILLARY DYSENTERY

Dysentery is an inflammatory condition of the intestine, mainly of the large intestine, characterised by necrosis, sloughing and ulceration of extensive areas of the mucosa with the presence of blood and mucus in the faeces It may be divided into two main types, amœbic and bacillary. The former, which is due to a protozoon, the *Entamœba histolytica*, will not be further considered here.

Before dealing in detail with the considerable number of bacteria capable of causing bacillary dysentery, we propose to discuss such general questions as the spread of the organisms, their distribution in the body, methods of diagnosis and treatment

All the species of the genus *Shigella* are strict parasites, their habitat being the human intestinal canal. A few have been recorded as causing dysentery in dogs but this is exceptional. It is not possible to produce a condition resembling dysentery in experimental animals by feeding, but inoculation of cultures of one species (*Sh. shiga*) may cause a somewhat similar condition in rabbits.

During the acute stages of dysentery, the causative organisms are excreted in the faeces in large numbers and, by hands, flies or other agents, may be conveyed to food Dysentery is less commonly spread by water than is enteric fever. The organisms may continue to be excreted in the faeces for long periods after convalescence The condition is then usually one of chronic dysentery.

The only reliable method of laboratory diagnosis is the examination of the faeces. Although the causative organism cannot be identified by microscopic examination, this method should not be

neglected because it shows, with a high degree of reliability, whether the condition is bacillary or amœbic dysentery. In acute bacillary dysentery 90 per cent. of the cells present in the motions are polymorphonuclear leucocytes while these cells are in a minority in amœbic dysentery.

For the identification of the species of *Shigella* responsible for the disease, a culture must be made from the feces. It is advisable to culture as soon as possible after a motion is passed. If this is impossible, a specimen of the feces may be preserved for a few days in buffered glycerol saline (see Chapter XLVIII). A satisfactory specimen of feces for culture may be obtained by means of a rectal swab or, still better, a swab taken from an ulcer with the aid of a sigmoidscope. A random loopful of feces spread on the surface of suitable medium usually yields colonies of the causative organism but a higher proportion of successful isolations will be achieved if a flake of mucus, preferably bloody, is selected with a platinum loop and washed in two changes of sterile saline before plating.

Formerly MacConkey's agar was commonly used for culture but it should now be discarded in favour of desoxycholate-citrate agar which gives a much higher proportion of positive results. Wilson and Blair's medium, tetrathionate broth and selenite broth should not be used as they suppress the growth of dysentery bacteria. After incubation of cultures on desoxycholate-citrate agar, small, clear, colourless colonies are picked, subcultured and examined by the usual cultural methods. Complete identification is achieved by agglutination tests using specific antisera.

The serum of a patient in the later stages of the disease frequently agglutinates the causative organism but, since the titre is not usually high, since normal serum may also cause agglutination and since such a variety of bacteria may cause dysentery, this method of diagnosis is not recommended.

Formerly antisera were used in the treatment of bacillary dysentery. The results obtained in the case of disease caused by

treatment of dysentery caused by other species was disappointing. Good results are now obtained by the use of sulphaguanidine or succinyl sulphathiazole. Since the organism is confined to the intestine, a relatively insoluble drug, such as those mentioned, is to be preferred to one which, being more soluble, is readily absorbed from the intestine.

Shigella

Organisms of this genus are Gram negative, non-sporing, non-motile, non-capsulated rods, usually measuring from 2μ to 3μ by 0.5μ to 0.7μ . They are aerobic and facultatively anaerobic. They ferment glucose, lactose, sucrose, mannitol, and salicin, usually without gas. They liquefy gelatin. The species of the genus is their ability to cause dysentery in human beings.

The bacteria of this genus are difficult to classify in a logical manner owing mainly to the occurrence of varieties which, on one basis, should be included in a species and, on another, excluded from it. Classification is based partly on biochemical activity and partly on antigenic structure. In connection with the latter, it

the different species. We have used names based on those of their discoverers or others who have contributed to our knowledge of the organisms.

A satisfactory and generally recognised method of division of the genus into two groups is according to the ability of these bacteria to ferment mannitol. On this basis we recognise a non-fermenting group and a fermenting group. In the mannitol fermenting group there are two well-defined species—*Sh. flexneri* and *Sh. sonnei*. In the mannitol fermenting group *Sh. flexneri* and *Sh. sonnei* constitute reasonably clear cut species. A good case could be made for introducing a new name and species, *Sh. boydii*, for the group of organisms which Boyd distinguished from *Sh. flexneri*.

NON-MANNITOL FERMENTING SHIGELLÆ
Shigella shigæ (*Shigella dysenteriae*) *
 Shiga, 1898

This organism has the characteristics common to the genus given above. It ferments glucose only, acid but not gas being produced. It does not produce indole.

It is the cause of the most serious type of dysentery which, in some epidemics in the past, had a fatality rate of 20 per cent. It is the only species of the genus which produces a powerful exotoxin. This toxin acts both on the intestine and on the central nervous system which explains the occurrence of paralysis after recovery from the acute stages of the disease.

The species is antigenically homogeneous and the identification of strains by agglutinating sera presents no difficulties. An anti-

Shigella schmitzii (*Shigella ambigua*)
 Schmitz, 1917

This organism resembles *Sh. shigæ* in fermenting glucose only without gas production but differs from it in producing indole.

Freshly isolated strains have two antigens, one of which may be lost on continued culture. For the preparation of agglutinating sera fresh strains should be used. The antigenic overlap of *Sh. schmitzii* and *Sh. shigæ* has been referred to already.

This is a rare cause of dysentery.

MANNITOL FERMENTING SHIGELLÆ
Shigella flexneri (*Shigella paradysenteriae*)
 Flexner, 1900

Organisms of this species ferment glucose and mannitol producing acid without gas. The majority produce indole.

Reasonably uniform biochemical activity and antigenic pattern of strains amply warrant the recognition of this species.

The organisms all contain an identical antigen which may be

referred to as the Flexner species antigen. In addition, each strain, when freshly isolated, possesses a type antigen. In freshly isolated strains, the type antigen masks the group antigen so that such an

has lost its type antigen completely, only by group serum. On

There is no difficulty in including in the species eight of the nine types but one (No. VI) presents difficulties because it is included on antigenic but not biochemical grounds.

Three strains (formerly known as Newcastle, Manchester and 88) constitute Sh. flexneri Type VI. Each of them possesses the

and a type antigen which is identical in
88

there-
arises

reasonable alternative to leaving the three strains in Type VI.

Sh. flexneri is a common cause of dysentery, often of a fairly mild type.

Shigella dysenteriae (Boyd) ~~TX~~

A group of bacteria differentiated from Sh. flexneri by Boyd is temporarily known as Shigella boydii. The main objection to this is the lack of any common antigen in the strains to be considered.

Biochemically they closely resemble typical Sh. flexneri, fer-

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Reasonably uniform biochemical activity and antigenic pattern of strains amply warrant the recognition of this species.

The organisms all contain an identical antigen which may be

glucose, maltose, mannitol and saccharose without gas production. It does not produce indole. The species is antigenically homogeneous, the only type of variation observed being the
smooth to rough variation already mentioned

occurred within 24 hours of the consumption of the food.

menting glucose and mannitol without gas formation. They do not produce indole.

Antigenically they are heterogeneous, not possessing any antigens in common with *Sh. flexneri* or with one another. Up to the present, four types (I to IV) have been discovered.

Organisms of this group, which are not common, have been isolated chiefly in India.

Shigella sonnei

Sonne, 1915

The type of growth of this organism on desoxycholate-citrate agar does not differ from that of other shigellæ. On MacConkey's agar, however, S → R variation occurs with unusual rapidity so that, even after overnight incubation, many strains, on primary isolation, produce a type of colony which, although predominantly smooth, contains a fan-shaped segment, the surface of which is rough and the edge of which extends beyond the circumference of the smooth part of the colony. The appearance of this type of colony is said to resemble that of a bomb crater. Colonies of other strains may at first appear quite smooth but become rough on the surface with irregularly crenated edges and the development of

variant which does. While a varying number of rough colonies always develop from the plating of a smooth colony, smooth colonies are never found when a rough colony is plated. The organisms in a rough colony are antigenically distinct from those in a smooth colony and, since either type may occur on first isolation
used for

It has been stated that only

lactose, *Sh. sonnei* ferments

bacteria of various species. This property was formerly attributed to the action of an enzyme, pyocyanase, but recent work suggests that it is due to a yellow pigment, x-oxyphenazine.

Ps. pyocyanea is widely distributed, being found in the faeces and on the skin, apparently as a harmless saprophyte, as well as in water and soil. It is a common secondary invader of diseased tissues, and is met with in the upper respiratory tract and in dirty wounds, in which it may play an important part in delaying

In these it has produced abscesses in various parts of the body, otitis media, peritonitis, pericarditis, empyema and bronchopneumonia. It is occasionally the cause of infection in the urinary tract. It may be responsible for some cases of gastro-enteritis in children and has been found in the blood stream in septicæmia. The lesions caused by it have characteristically a discharge which is green or blue in colour.

Proteobacteriaceae -

Proteus - { occurring in various distinct forms.

Bacteria of the genus *Proteus* are highly pleomorphic, varying in length from almost coccal forms to very long filaments which are frequently curved. Such filamentous forms are particularly associated with the swarming type of growth referred to below. The organisms of the genus are actively motile, non-capsulated, non-sporing and Gram negative. They have peritrichous flagella.

Probably the most characteristic feature of the genus is the peculiar type of growth called swarming which, under suitable conditions, all the members of the genus exhibit.

off from the edge of the culture to invade fresh areas. In this way, usually within 24 hours, the whole surface of the medium is covered with a thin, almost transparent layer of bacteria which may easily be overlooked. On account of this characteristic, it is

CHAPTER XXXIII

MISCELLANEOUS BACTERIA

Pseudomonas pyocyanea (*Pseudomonas aeruginosa*)
Schröter, 1872

Ps. pyocyanea, which measures from 1.5μ to 5.0μ by 0.3μ to 0.6μ , is found most commonly in pairs, but single bacilli and short chains frequently occur. The organism is actively motile, has no capsule and does not form spores. It is Gram negative.

It grows readily on ordinary media at body temperature under aerobic conditions, but growth can also take place at the temperature of the air and in the absence of oxygen. The outstanding feature of this organism is the production of two pigments, one of which, called pyocyanin, is of a blue colour and the other, fluorescein, is greenish-yellow and fluorescent. For the production of the colour the presence of oxygen is essential. After some time the colour darkens, and the medium may become dark reddish-brown or almost black. On agar a greyish, moist, glistening growth with a metallic sheen is produced, which shows very little if any of the characteristic colour on the surface, since the pigments diffuse through the medium, giving to it the greenish-blue colour. Gelatin is rapidly liquefied. In broth, which becomes only slightly coloured, a turbidity is produced with the formation of a pellicle and a flocculent deposit. *Ps. pyocyanea* does not ferment

of glucose.

coagulated

filtrate of an

of killing

bacteria of various species. This property was formerly attributed to the action of an enzyme, pyocyanase, but recent work suggests that it is due to a yellow pigment, x-oxyphenazine.

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In these it has produced abscesses in various parts of the body, otitis media, peritonitis, pericarditis, empyema and broncho-pneumonia. It is occasionally the cause of infection in the urinary tract. It may be responsible for some cases of gastro-enteritis in children and has been found in the blood stream in septicæmia. The lesions caused by it have characteristically a discharge which is green or blue in colour.

Proteobacteriaceae:— *Proteus* { occurring in various distinct forms.

Bacteria of the genus *Proteus* are highly pleomorphic, varying in length from almost coccoid forms to very long filaments which are frequently curved. Such filamentous forms are particularly associated with the swarming type of growth referred to below. The organisms of the genus are act non-sporing and Gram negative.

Probably the most characteristic peculiar type of growth called swarming occurs under suitable conditions all the species in the genus display this characteristic only on over the

In this way, the medium is bacteria which may easily be overlooked. On account of this characteristic, it is

sometimes very difficult to isolate pure cultures of other bacteria, such as streptococci, from material containing a *Proteus*. Various methods have been suggested of which the best is probably the medium: gelatin.

fermentation (with gas production) of glucose and usually of saccharose but not of lactose, mannitol or dulcitol, the active decomposition of urea and the production of H_2S .

Proteus species are commonly found in the soil, in decomposing organic matter and in water. They are associated with putrefaction but, growing rather feebly under anaerobic conditions, not with its later stages which are effected mainly by anaerobic organisms. ("Bacterium of putrefaction" = *Bacterium termo*).

Bacteria of this genus are not infrequently found in normal

found in contaminated wounds and appear to be responsible for delayed healing in them. Some species, and especially *Pr. morganii*, are found in large numbers in the faeces of infants suffering from gastro-enteritis but their aetiological connection with the condition is doubtful.

The two species most commonly found about the human body are *Pr. vulgaris* and *Pr. mirabilis*. The former ferments glucose, maltose and saccharose, the latter glucose and saccharose but not maltose. *Pr. vulgaris* produces indole, *Pr. mirabilis* does not. Morgan's No. 1. Bacillus: —————>

Pr. morganii differs from the majority of *Proteus* species in failure to produce H_2S . It ferments glucose only. It produces indole. It does not swarm on ordinary moist agar at 37° but on soft moist agar at 20° - 28° , characteristic swarming occurs.

Certain strains known as *Proteus X₂*, *Proteus X₁₉* and *Proteus*

the various strains the same is determined by an alkali-st. the carbohydrate
this is also found in the *Rickettsia prowazekii*. The strains X 19,
which ferment Maltose.
MISCELLANEOUS BACTERIA 357

with alcohol to destroy the flagellar antigens should be used.

Voges-Proskauer reaction negative but the methyl red
is positive. *Pr. Morganii* are actively proteolytic. Nitrate
reduced.
Alcaligenes faecalis
(*Bacillus*)

This is a short, motile, Gram negative, non-sporing bacillus
which is found singly, in pairs or chains. It does not liquefy
gelatin, produce indole or ferment any carbohydrate. It is a
normal inhabitant of the intestine and may be recognised on Mac-
Conkey plates prepared from faeces by the yellowish zone sur-
rounding the colonies, which is due to alkali production. It has
feeble pathogenic power. Causes infantile diarrhoea but
infection

Lactobacillus

Bacteria of the genus *Lactobacillus* are mostly long, slender rods
which are Gram positive, non-motile, non-capsulated and non-
sporing. They are, for the most part, microaerophilic or anaerobic.
Although they grow best in media of neutral reaction, they are
aciduric, that is they are capable of growing in suitable media of
such a high hydrogen ion concentration as to prevent the growth
of most other bacteria, a feature which greatly facilitates their
isolation. They ferment a variety of carbohydrates, rarely produc-
ing gas. They do not liquefy gelatin.

The most frequently occurring species in relation to the human
body is *L. acidophilus* which constitutes the majority of the
bacterial flora of the faeces of breast fed infants. It can usually be
isolated from the faeces of adults although, where a normal diet
is consumed, the number present is small. This organism is
commonly used to sour milk intended for therapeutic purposes.
If milk so soured is consumed in considerable quantities together
with fresh milk and lactose, the faecal flora is transformed into one
consisting chiefly of *L. acidophilus*, proteolytic bacteria being

inhibited by the acid reaction resulting from the fermentation of lactose.

L. bulgaricus was formerly used for the same purpose, but less successfully. As the organism does not grow in the intestine, the individuals found in the faeces are merely those which were swallowed.



FIG. 62.—*L. acidophilus* FROM MILK CULTURE.

Another species, *L. bifidus*, also found in infant faeces, can be distinguished from *L. acidophilus* by the occurrence of clubbed and branched forms.

✓ Lactobacilli, either *L. acidophilus* or closely related species, occur in a variety of situations about the human body in addition to the intestinal canal. Among these may be mentioned the teeth where the organism, believed by some to be a factor in the causation of dental caries, has been called *L. odontolyticus*;
 ✓ the vagina the acidity of which is, in part at least, attributable to the organism, in this situation called Döderlein's bacillus; and the stomach, in which the organism, under the name of the Boas-Oppler bacillus, occurs when, as in gastric carcinoma and pyloric obstruction, the hydrochloric acid is reduced in amount.

Except possibly in the case of the teeth, which may be injured by the acid produced by the *Lactobacillus* from carbohydrate foods lodging there, bacteria of this genus are beneficial rather than harmful.

CHARACTERISTICS OF CERTAIN INTESTINAL BACTERIA

Organism		Motility	Fermentative Reactions						Indole production
Genus	Species		Lactose	Glucose	Maltose	Mannitol	Saccharose	Dulcitol	
BACTERIUM	<i>coli aerogenes</i>	+	AG	AG	AG	AG	-	AG	+
		-	AG	AG	AG	AG	AG	-	-
SALMONELLA	<i>typhi</i>	+	-	A	A	A	-	-	-
	Other species	+	-	AG	AG	AG	-	AG	-
SHIGELLA	<i>shiga</i>	-	-	A	-	-	-	-	-
	<i>schmitzii</i>	-	-	A	-	-	-	-	+
	<i>flexneri</i>	-	-	A	A or -	A	-	-	+ or -
	<i>sonnei</i>	-	A (late)	A	A	A	A (late)	-	-
PROTEUS	<i>vulgaris morganii</i>	+	-	AG	AG	-	AG	-	+
		+	-	AG	-	-	-	-	+

+ = motile or indole produced

- = non-motile, no indole produced or no change in reaction

A = acid only

AG = acid and gas.

CHAPTER XXXIV

Prepared by the U.S. Navy

Vibrio cholerae (*Vibrio comma*)

Koch, 1884

The cholera vibrio measures from 1.5μ to 2.0μ by about 0.5μ . As seen, usually in enormous numbers, in the dejecta from cases of cholera, it is definitely curved, and, from its resemblance to a comma, the name "Comma bacillus" was given to it. The curve, however, is not flat, but is in two planes. Frequently the organisms occur in pairs, giving either a C or, more commonly, S shape

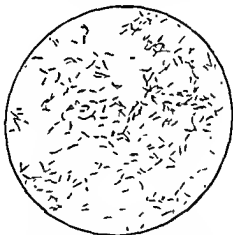


FIG 63—*V. cholerae* FROM AGAR CULTURE ($\times 950$).

Only rarely are long spiral chains found in the intestinal contents. Where a film is made from a small flake of mucus in the watery discharge the axes parallel, in a stream. In cultures—particularly of old laboratory strains—the curved shape may be almost or completely lost, and the organism then seems to resemble a coliform bacillus. Old cultures may

how large, badly stained involution forms which are commonly clubbed or coccal in shape. In fluid media long spiral chains of vibrios are of frequent occurrence. The cholera vibrio stains with the ordinary stains less easily than the majority of other bacteria and is Gram negative. The vibrio is actively motile having a single terminal flagellum. It has no capsule and does not form spores. *Ching's test*

The organism grows best aerobically. under strictly anaerobic conditions very poor growth is obtained. It is not very restricted as regards the type of medium used, but prefers an alkaline reaction, it will grow readily on media sufficiently alkaline (pH 9.0 to 9.5) to prevent the development of the majority of other pathogenic bacteria. It grows well at both air and body temperature (16° to 42° — optimum growth at 37°C)

On agar the colonies are round, thin, very transparent and almost colourless. In old cultures however the agar frequently

the organism's growth in media containing glucose, mannitol, maltose, saccharose and mannose. Neither dulcitol nor arabinose is fermented. The majority of true cholera vibrios are non-hæmolytic. (No production of soluble hæmolysin)

The resistance of the vibrio to heat resembles that of other non-sporing bacteria, but it is very susceptible to drying, being thus

in rivers, it does not usually survive for more than a fortnight.

Cholera in man is an acute disease in which, in the majority of cases, the causative organisms are confined to the intestine,

p-tube a yellowish brown pigment is formed by
serology. Pfeiffer's Phenomenon — (agglutination and
 ... ultra-benitoneally into culture an inv.

although the body as a whole suffers from the most intense toxæmia, as shown by the subnormal temperature and the profound collapse and prostration. The lower half of the small intestine is the region most affected. The wall is congested and, owing to the penetration of the cholera vibrios between and beneath the epithelial cells, the latter are loosened and desquamated. The content of the gut is watery, and in it float flakes composed of masses of separated epithelial cells and of mucus; the common term "rice water stool" is a fairly good description of the patient's motion. The intense diarrhœa, which is accompanied by severe cramps, dehydrates the body to such an extent that the blood is concentrated and the urine is very scanty. Where the disease is more chronic, there may be extensive necrosis of

the gall-bladder and occasionally in lymph nodes, spleen, kidneys and elsewhere. (Cholera) - Vibrio in P. 100 and 101

Among the outstanding features of the disease are: (1) its short incubation period, usually 1 or 2 days; (2) its rapidity of development, death frequently occurring within 12 hours of the onset of the disease; and (3) the intense toxæmia. The first two of these are explained by the rapid rate of growth of the cholera vibrio, whether within the body or in vitro. The third feature is due to the intense toxicity of the organism itself. Most workers have found that filtrates of young broth cultures are but very slightly toxic, that is, an extracellular toxin is either absent or occurs only to a small extent. The most important toxin is the endotoxin

..... To it are due the toxic

..... disease, and by animals
..... id. The liberation of the
toxin in the bodies of man or of animals is due largely to the
bacteriolytic action of serum on the vibrios. The toxin is found
in filtrates of old broth cultures owing to autolysis, and can be
liberated artificially by grinding up cultures when frozen.

An enormous amount of work has been done to elucidate the problems of cholera experimentally. That the cholera vibrio is the cause of the disease has been proved by several experiments on man, some intentional and some accidental, in which the swallowing of pure cultures has occasioned the disease. Others, however, have escaped, and it seems probable that in order to give the assistance of the

—cor... vibrios in their stools—are known, and that they may, possibly as a result of some dietary indiscretion, acquire the actual disease. It is remarkable how often the layman attributes cholera to indulgence in over-ripe fruit.

No animal, under natural conditions, suffers from cholera, but, by artificial procedures such as neutralisation of the gastric juice with sodium bicarbonate, it is possible to produce in experimental animals a condition closely resembling human cholera. Intra-peritoneal injection of the guinea-pig with cholera vibrios, whether living or dead, produces peritoneal effusion, subnormal temperature, collapse and death.

man or animal is found to contain antibodies to the organism. It is very slightly, if at all, antitoxic, as can be well shown by the intraperitoneal injection into a guinea-pig of a culture of cholera vibrios followed by immune serum. Owing to the rapid lysis of the vibrios and the consequent liberation of their endotoxin, the animal dies more rapidly than another which has received a corresponding dose of culture without serum.

During the course of cholera enormous numbers of vibrios are excreted in the faeces and the disease is spread by the contamination of water supplies or food with such excreta. The vitality of the organism outside the body is not great, but the existence of the disease is maintained by carriers. The carrier state does not

agglutination test. Agglutinins appear early in the path...

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the gall-bladder and occasionally in lymph nodes, spleen, kidneys,
lungs and elsewhere. (Cholecystitis) - Vibrios in Bile and Gall

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liberated artificially by grinding up cultures when frozen.

Structure of Vibrios: (Three types of Polysaccharides are
seen. Based on the hydrolysis products of Polysaccharides)

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Structure of Vibrios. (Three types of Polysaccharides +
protein, Based on the hydrolysis products of Polysaccharides)

CHAPTER XXXV

PASTEURELLA

Pasteurella pestis

Yersin and Kitasato, 1894

Past. pestis is found in its most characteristic form in material taken from early plague lesions in man or animals. It is short and thick, with rounded ends, and may frequently appear almost coccal in outline. Its average size is from 1μ to 2μ by 0.5μ to 1.0μ , but under certain conditions much larger forms are also found. It occurs in the body either singly or in pairs, short chains being exceptional. Young cultures on solid or in fluid medium present a very similar appearance, but there is a tendency for the organism to become more definite when grown on solid media.

In cultures a

occurrence.

agar" (agar containing about 3 per cent sodium chloride) show

feature of the bacillus is the occurrence of polar staining. The central part of the bacillus is unstained, or but faintly stained, while each pole takes the stain intensely. By continued artificial culture this appearance is, to a considerable extent, lost. *Past. pestis* possesses a capsule which, however, is not always easily demonstrable. It is non-motile and forms no spores.

→ The plague bacillus grows best under aerobic conditions: in the complete absence of oxygen growth is either absent or very slight. The optimum temperature of cultivation is about 30° , but growth occurs quite satisfactorily at body temperature. It grows readily

usually last for more than a fortnight after convalescence, and its existence for 2 months is uncommon, chronic carriers being exceedingly rare. Healthy carriers are a great danger both to themselves and to others.

* A strain of cholera vibrio known as the "H₁ T₁ strain"

and cultural characteristics, but is actively hemolytic and produces an extracellular toxin. It is agglutinated and dissolved by anti-cholera serum. Most observers now regard it as a modified cholera vibrio. Many other vibrios, of which some cause disease in lower animals and others are non-parasitic, are known.

The diagnosis of cholera in man can frequently be made by direct microscopical examination of the fluid stools, both stained and unstained; the occurrence of large numbers of typical vibrios is almost pathognomonic. The vibrios can be isolated by adding

will usually show large numbers of vibrios; but if necessary a second inoculation may be made from the first, and in the case of carriers even a third may be required. The very rapid surface growth of cholera vibrios secures their enrichment and they can then be isolated by plating a loopful from the surface on alkaline agar or a special alkaline blood-agar, Dieudonné's medium.

Strict criteria are required for the identification as *V. cholera* of a *Vibrio* isolated from the faeces of a suspected carrier or from a sample of water.

cholerae are ferment. arabinose, and the ch.

teristics may be confidently identified as *V. cholerae* if it is agglutinated by a specific anti-cholera serum. *V. cholerae* possesses both H and O antigens, many of which occur in other, non-pathogenic,

human beings by the use of cholera vaccine.

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Yersin and Kitasato, 1894

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agar" (agar containing about 3 per cent. sodium chloride) show many involution forms, large, faintly staining, ovoid, globular, or club-shaped, somewhat suggestive of yeasts.

The organism stains readily with ordinary stains, but is Gram negative. When freshly isolated from the body, a characteristic feature of the bacillus is the occurrence of polar staining. The central part of the bacillus is unstained, or but faintly stained,

complete absence of oxygen growth is either absent or very slight. The optimum temperature of cultivation is about 30°, but growth occurs quite satisfactorily at body temperature. It grows readily

on any of the ordinary laboratory media, but the first cultures made from the body may require an enriched medium, such as blood-agar. On agar the colonies are small, translucent, with granular centres and somewhat irregular edges. A culture on an agar slope is translucent and the growth, when touched with a platinum wire, is sticky. In older cultures a certain irregularity in the size of the colonies, almost suggestive of impurity in the culture, is rather characteristic. Gelatin is not liquefied by the growth of *Past. pestis*. When grown in broth the organisms fall to the

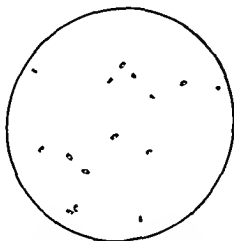


FIG. 64.—*Past. pestis* FROM YOUNG AGAR CULTURE ($\times 950$).

bottom of the tube as a granular deposit, leaving the upper part clear. If, however, a little oil is floated on the broth, the growth starts from the lower surface of the oil and long delicate filaments, composed of masses of bacilli, grow downwards towards the bottom. To secure this stalactite type of growth it is essential that the flask should remain absolutely steady, as the slightest movement is sufficient to detach the threads. In broth the bacilli occur most commonly in long chains. No indole is produced by the growth of *Past. pestis* in broth or peptone water. The organism produces acid without gas in media containing

glucose, mannitol and usually maltose. No fermentation of lactose, saccharose or dulcitol is found.

The resistance of the organism against heat, drying, and antiseptics is not especially marked. It withstands, however, a consi eks in

dried Pla of the
rat te and

rapidly fatal septicæmia. The lymphatic glands, which are enlarged and congested, are surrounded by hæmorrhagic and œdematous areas. The spleen is enlarged and the liver is mottled owing to the occurrence of small areas of necrosis alternating with hæmorrhagic points. There is generally an excess of fluid in the pleural cavities. More rarely plague may take a more chronic course in the rat. A rat usually becomes infected by the bite of a flea which has previously bitten a rat suffering from plague septicæmia. Infection also occurs by devouring of the carcass of a rat dead of the disease.

Human plague is a most serious disease, and from time to time there have been widespread epidemics causing the deaths of millions of victims. It may occur either in mild form, Pestis Minor, in which a moderate pyrexia with slight enlargement and tenderness of one or more groups of lymphatic glands are the chief characteristics, or as Pestis Major, which may assume one of three types: Bubonic, Pneumonic, or Septicæmic. ✓Bubonic plague, which is of commonest occurrence, commences with a progressive swelling of a group of lymphatic glands, most frequently the inguinal, accompanied by great pain. ✓Marked pyrexia and great prostration are two of the outstanding characteristics of the disease. The pathological condition of the swollen glands is one of intense inflammation with hæmorrhages, leading eventually to necrosis. Not only the glands themselves but also

tissue. In the early stages the glands are packed with enormous numbers of coccoid, polar-staining bacilli, and a film prepared

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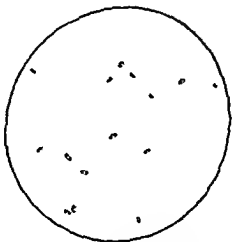


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spreading broncho-pneumonia of a hæmorrhagic type. Enormous numbers of the bacilli are to be found in the bloody sputum. This form of " ", since the

Stream is early invaded,
in the glands or lungs.

Laboratory animals, particularly guinea-pigs, rats, and mice, are susceptible to infection. As a result of subcutaneous injection in the guinea-pig, there is a local lesion with marked œdema, congestion, and small hæmorrhages. The glands draining the area involved are enlarged and their condition is very similar to that found in human plague. Congestion and hæmorrhages are found in the internal organs, particularly in the spleen, which is often studded with small pale areas of necrosis resembling, in naked eye appearance, miliary tubercles. The animal generally dies within a week of receiving the injection. The guinea-pig may also be infected by smearing the conjunctiva, or the nasal mucous membrane, or by rubbing the freshly shaved skin of the abdomen

Final method

, such as

Que. Rats

and mice may also be infected by eating food containing the bacilli.

Infection of man in the bubonic and septicæmic types occurs through the skin, either owing to the bacilli contained in dust finding entry through minute abrasions or, in the majority of cases, from the bite of a rat flea which has previously fed on a plague rat. The bacilli in the blood of the rat multiply in the stomach of the flea and are regurgitated at the next feed. The bacilli are also present in the flea's faeces, and by scratching the site of the bite on which the insect has defæcated, the bacilli may be forced into the wound and so enter the body. A flea may remain infective as long as 3 weeks after partaking of blood from a plague rat. In India, the common host of *Past pestis* is the rat. The disease is spread from rat to rat by the rat flea. As a result of an epizootic of plague among rats, the number of these is reduced

from the gland pulp may resemble a pure culture of the organism.

When necrosis becomes marked, the number of bacilli decreases

h does not supervene

at a slightly later date

i. In addition to the

primary bubo, one or more secondary buboes may appear in other groups of glands. These are usually much less marked, but they show changes similar to those of the primary bubo. The spleen is enlarged and may show areas of hæmorrhage and

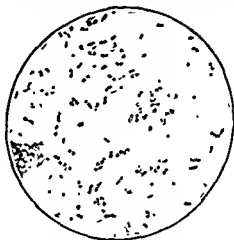


FIG. 65.—*Past. pestis* IN FLUID FROM BUBO ($\times 800$).

necrosis, as may also the liver and lungs. In the spleen, as in the glands, large numbers of bacilli are present. The cells of the liver and sept. num

found by direct microscopical examination. The bacilli are frequently present in the blood, even at an early stage of the disease, but in much smaller numbers, and this does not portend, of necessity, a fatal issue. The mortality of bubonic plague varies in different epidemics from 20 to 90 per cent.

(8) In pneumonic plague, the condition is primarily one of rapidly

tion of *Past. pestis*, the outstanding points are characters of growth on agar, in broth, and on salt agar, microscopical appearance, biochemical activity, agglutination with specific serum and pathogenicity for animals

The examination of carcasses of rats and of other animals which have died from a plague-like disease requires isolation of the organisms and their complete investigation. Where putrefaction has occurred ordinary cultural methods usually fail, but *Past. pestis* can be isolated by making use of the fact that the organism can penetrate the nasal mucous membrane or the shaved skin of the guinea-pig, the majority of other bacteria failing to do so.

The antigenic similarity of *Past. pestis*, *Past. pseudotuberculosis* and *Past. septica*, and particularly of the first two, is so great as to render their serological differentiation very difficult. The results of serological investigation should be interpreted with caution and with due consideration for biochemical activity and pathogenicity for animals.

Pasteurella pseudotuberculosis

Mallassez and Vignal, 1883

Past. pseudotuberculosis is very similar in general characteristics to *Past. pestis* and antigenically the two are almost identical. Points in which *Past. pseudotuberculosis* differs from *Past. pestis* are the fact that it is motile when grown at 20°-22° and that all strains ferment maltose and some saccharose.

The organism is not believed to be pathogenic for human beings but is for a large variety of animal species. It is the cause of naturally occurring disease in rodents, the acute form of which resembles plague. In the chronic form, caseous lesions which resemble those of tuberculosis occur in glands, spleen, liver and lungs.

Pasteurella septica (Pasteurella multocida)

Kitt, 1878

Past. septica is very similar to the other pasteurellæ but can be distinguished from them by its fermentation of saccharose but

great intermediary between the rat and man and preventive measures are to be directed chiefly to the elimination and exclusion of rats.

✓ In pneumonic plague, infection is usually direct from patient to victim by means of droplets of sputum containing bacilli. Sporadic cases of pneumonia occur in epidemics of bubonic plague, and these are probably the origin of epidemics of the pneumonic form of the disease. It seems possible that in a short time the organisms acquire a preference for the tissue of the lung

The toxins of *P*
of broth cultures

cultures of the bacillus, killed by heating to 65° for 1 hour, may cause areas of necrosis and hæmorrhages in the internal organs.

Since one attack of plague protects for life, attempts have been made to secure immunity in man by vaccines. The incidence of the disease has been lower in the vaccinated, and in those who did acquire it the mortality has been less, but the protection lasts for only a few months.

Sulphonamides and particularly sulphathiazole are of value in the treatment of plague and good results have also been reported with streptomycin.

The bacteriological diagnosis of the disease in man presents no special difficulties. In places where bubonic plague is endemic, a microscopical examination of fluid, aspirated from a bubo with a hypodermic syringe and needle, is usually satisfactory, as the finding of large numbers of short, fat, polar staining bacilli is sufficient to confirm the diagnosis. It should be noted, however, that in cases which recover, no bacilli may be found microscopically or on culture in the gland fluid in the later stages, although the bubo is still present. In the first case occurring in a non-plague locality and in the examination of the sputum from a case of suspected pneumonic plague, it is essential to isolate the organism and to investigate its characteristics. In the identifica-

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Pasteurella septica (Pasteurella multocida)

Kitt, 1878

Past. septica is very similar to the other pasteurellæ but can be distinguished from them by its fermentation of saccharose but

not usually of maltose and by its production of indole. Like *Past. pestis* it is non-motile.

It gives rise to a hæmorrhagic type of septicæmia in a large variety of birds and mammals. Strains were formerly given such specific names as *avisепtica*, *bovisепtica*, *lepisепtica* and *suisепtica* according to the animal species from which they had been isolated. These were sometimes referred to as constituting the hæmorrhagic septicæmia group of bacteria.

The organism occasionally causes abscess formation in man.

Pasteurella tularensis *Primarily a disease of Rodents → a fatal Septicæmia.*
McCoy and Chapin, 1912

Past. tularensis is smaller than *Past. pestis* and possesses a capsule. Primary cultures are rather difficult to obtain, medium containing blood, glucose, and 0.1 per cent. of cystine being most successful. The colonies are small and viscid.

This organism is responsible for a disease occurring naturally in ground squirrels, rabbits, hares and other rodents and possibly also in sheep, cattle, grouse, and quail. Mice, guinea-pigs, and rabbits can be infected experimentally. The disease in human beings, which is known as tularæmia, was first observed in America, but, later, cases were recorded in Japan, Siberia, Russia, Scandinavia, and Central Europe. Several bacteriologists elsewhere have been infected from lab take place by inoculation, through the conjunctiva, and possibly by animals suffering from the disease, of blood-sucking insects—the deer fly (*Chrysops discalis*), the wood tick (*Dermacentor andersoni*), and others which are responsible for its spread among animals.

In animals the disease closely resembles plague and is very fatal, but in man it is milder and rarely fatal, although of long duration. It may resemble typhoid fever or present, as its most striking features, a primary ulcer at the site of inoculation and glandular enlargements which may progress to suppuration.

Direct cultures from human cases have usually been unsuccessful. The best method of isolating the organism is to inject a guinea-pig intraperitoneally with material from the human lesion and obtain a culture from the necrotic foci which develop in the liver, spleen and lungs of the animal.

Very successful results have been obtained in cases of tularæmia by treatment with streptomycin. ✓

CHAPTER XXXVI

HÆMOPHILIC BACTERIA

Hæmophilus Influenzæ

Pfeiffer, 1892

The influenza bacillus of Pfeiffer is one of the most minute of the ordinary bacteria, measuring from 1.0μ to 1.5μ by 0.2μ to 0.3μ , but almost coccal forms are common in body fluids and filaments may occur in culture. It is found in very large numbers in the sputum of many patients in the early stages of the disease, but experienced observers have failed to detect its presence after careful search in a considerable proportion of cases of clinical influenza. When present, it is found lying singly, in pairs, or most commonly in clusters but never in chains, either between or within the leucocytes in the sputum. It is non-motile and does not form spores. Fully virulent strains are capsulated. It is Gram negative and stains less readily than the majority of bacteria, the most successful stains being dilute carbol fuchsin or alkaline methylene blue, applied for a longer time than usual. Frequently the bacilli show bipolar staining, the central portion taking the stain less intensely than the ends.

The bacillus is strictly aerobic and will grow only at, or near, body temperature. It will not grow on plain or serum agar. On blood-agar colonies are just visible as very minute, clear, drop-like dots at the end of 24 hours. On the next day the colonies may have a diam. fuse growth the tubes of

100° for a few minutes. On this medium the colonies are much larger, grey in colour, and more opaque.

Two factors, X and V, are necessary for the cultivation of *H. influenza*. X, in blood-agar or chocolate agar, is hæmin derived

animal and vegetable tissues, in blood and yeast, has been identified as one of the two co-dehydrogenases, di- or tri-phosphopyridine nucleotide. It is also present in cultures of most bacteria, and its production by staphylococci is illustrated by the phenomenon of satellitism, so characteristic of *H. influenza*. This is the great increase in the size of colonies of the organism on blood-agar in the vicinity of a staphylococcus colony.

H. influenza ferments glucose and sometimes, also, maltose and saccharose, acid only being produced. Neither lactose nor mannitol is fermented. Some strains produce indole and some are hæmolytic.

The pleomorphism of *H. influenza* has been referred to already. Its morphology is fairly closely correlated with colony form and antigenic structure. The fully virulent organism is almost coccal in morphology, is capsulated and produces smooth, slightly mucoid colonies. When capsules are no longer produced, the organism assumes a more definitely bacillary form and the colonies are smooth but not mucoid. This is the type most commonly isolated from the respiratory tract. Relatively non-

sputum and in cultures its vitality does not persist for more than a few days. It is easily killed by heat and antiseptics.

The organism is pathogenic for animals but in none is it possible, by inoculation of cultures, to produce a disease having any resemblance to human influenza. By direct injection into the lungs of monkeys an acute respiratory disease with pyrexia and broncho-pneumonia may be produced.

That Pfeiffer's bacillus was the cause of influenza in man was commonly accepted until the epidemics of 1918 and 1919. During these years many bacteriologists, in many lands, investigated the bacteriology of the disease. Some, in certain places, found the bacilli in practically every case. Others, however, failed in many or even in the majority of cases to isolate the organism. Experi-

brane of the respiratory tract.

It is now universally accepted that influenza is due to a virus. In some epidemics, however, a coincident infection with a virulent strain of *H. influenza* makes the disease more serious and more fatal. It is of interest to note that the influenza of swine is due to the combined action of a virus, closely resembling the virus of human influenza, and of *H. suis*, an organism very similar to *H. influenza*.

Apart from influenza, *H. influenza* is definitely pathogenic for man. It causes a not uncommon variety of meningitis and a rather rare type of Koch-Weeks bacillus.

it should now be called by that name. *H. influenzae* is commonly found in the sputum in acute and subacute infections of the respiratory tract.

H. influenzae is relatively resistant to penicillin, a fact which may be used to facilitate its isolation from sputum. When penicillin is either incorporated in the medium or is spread over its surface, the growth of most pyogenic cocci, but not of *H. influenzae*, is prevented. Its resistance is not, however, absolute and when penicillin can be applied locally in high concentration, as in meningitis, good therapeutic results are obtained. It is sensitive to streptomycin.

Hæmophilus pertussis

Bordet and Gengou, 1906

H. pertussis is an organism very similar to Pfeiffer's bacillus. It is inclined to be more definitely bacillary than that organism which it resembles in staining peculiarities and in characteristic grouping. It is normally capsulated, non-motile, does not ferment any sugar and does not produce indole.

It is more difficult to obtain in primary culture than *H. influenza* and, for culture, it is essential. early stages of whooping cough numerous, disappear later.

and Gengou, which is potato-extract glycerol-agar with up to 50 per cent. of blood, is superior to plain blood-agar. The colonies after 72 hours incubation are smaller than those of Pfeiffer's bacillus but otherwise similar. Sub-cultures may, however, grow much more luxuriantly than those of *H. influenza*, the colonies becoming sticky, opaque and grey in colour with a dull metallic lustre like that of aluminium paint. Strains of the organism cultured for some time on artificial media can grow in the absence of both X and V factors and even on plain agar. It has been suggested that the high proportion of blood required in media used for the culture of *H. pertussis* is of value on account of the albumin, rather than the hæmoglobin, which it contains. It is possible that, if this view be correct, the albumin acts as an absorber of some deleterious product of growth of the organism, such as oleic acid, rather than as a nutrient. Blood required for the growth of certain other bacteria, such as *H. ducreyi*, may serve the same purpose.

In the early stages of whooping cough, the bacilli may be found in enormous numbers in the sputum, particularly in the thick viscid secretion obtained from the smaller bronchioles at the end of a paroxysm of coughing. They are also seen in sections of the lung lying on or between the cells of the mucous membrane of the bronchioles and alveoli. When correct technique is adopted the organisms are found in almost every case of the disease. Plates of Bordet and Gengou's medium may be inoculated by spreading a specimen of sputum, by allowing the patient to cough

on the plate and so distribute droplets containing the organism over its surface (cough plate method) or, best of all, by spreading material collected with a swab from the posterior naso-pharyngeal wall. The addition of 10% (100 mg. per ml.) and of streptomycin inhibits the growth of which would render the

isolation of *H. pertussis* difficult. *H. pertussis* is distinctly toxic for some animals, the intravenous injection of autolysed cultures killing rabbits in from 24 to 48 hours. Only endotoxins are produced. Intra-tracheal inoculation of dogs and monkeys with the living organism produces a catarrhal inflammation of the respiratory mucous membrane, with pyrexia and occasionally patches of broncho-pneumonia, death occurring in a number of cases in from 2 to 3 weeks.

While the bacterium has not been definitely proved to be the causal organism of whooping cough, the evidence is largely in favour of this. The fact that it has been found in the sputum of a small number of adults suffering from chronic bronchitis is no weighty objection.

All recently isolated, smooth strains of *H. pertussis* are of the

learned up some of the difficulties in connection with the serology of *H. pertussis*. In artificial culture, on unsuitable medium, the acillus undergoes antigenic changes, passing from Phase I, the normal, smooth, fully virulent form, through Phases II and III to phase IV, in which the organism is non-capsulated, non-toxic and gives rise to rough colonies.

Vaccines are used prophylactically and appear to give a considerable degree of immunity. Three injections should be administered, preferably during the first 6 months of life. For their preparation organisms in Phase I must be used.

man, M. ... Incubation period is 2-14 days as the time ... after the ingestion of the ... disease is ... 381

BACTERIA CAUSING UNDULANT FEVER

381

Maximum growth is obtained on liver extract agar. In broth, slight turbidity and deposit are slowly produced. Gelatin is not liquefied, indole is not produced and no sugar is fermented.

The organisms resist cold and drying very well and survive in dry dust for some months. They have no remarkable resistance against heat or chemical antiseptics. Killed at 60°C in 10-15 minutes.

Before dealing with the pathogenic properties of the three species, the methods by which they may be distinguished from one another will be considered. In some cases differentiation is relatively easy, in others very difficult. Information as regards the locality and the species of animal from which the strain was isolated is of value.

The requirement of *Br. abortus* (except Rhodesian strains) for additional CO₂ in the atmosphere to which early cultures are exposed has already been mentioned. *Br. abortus* and American strains of *Br. suis* give off H₂S from cultures in considerable amount and for several days. *Br. melitensis* and Danish strains of *Br. suis* either give off no H₂S or a small amount for only 1 day. The inhibitory action of certain dyes incorporated in the medium (preferably liver extract) is probably the most valuable criterion for differentiation of the species. The most useful dyes are thionin and basic fuchsin. The optimum concentration depends to some extent on the medium used, but usually lies between 1/30,000 and 1/60,000 for thionin and between 1/25,000 and 1/50,000 for basic fuchsin. *Br. melitensis* grows in the presence of both dyes, *Br. abortus* in the presence of basic fuchsin but not of thionin and *Br. suis* in the presence of thionin but not of basic fuchsin.

Antigenically the organisms are very similar, while still smooth, they appear to possess two or more antigens which are common to the three species but occur in them in different proportions. In consequence of this, antiserum prepared against one agglutinates all three. By using the absorption of agglutinin technique, it is possible to distinguish *Br. melitensis* from the other two but it is

BACTERIA CAUSING UNDULANT FEVER (H. L.)

is septicæ :-	is a secondary	is a Crime distemper	(Micrococcus).	Gram Negative,
			i/ <i>Brucella melitensis</i>	rods with coccoid
			Bruce, 1887	non motile, non
			ii/ <i>Brucella abortus</i>	aerobic or micro
			Bang, 1897	Parasitic and path
			iii/ <i>Brucella suis</i>	for man
			Traum, 1914	Carbohydrates not

that it is doubtful if the granting of a species rank is warranted but, as a matter of convenience, we propose to refer to them by the names given above. $\text{size} = 0.5-1.5 \times 0.6 \mu$

These organisms are very minute rods which may be so short as to appear coccoid in morphology. In fact, *Brucella melitensis* was for some years called *Micrococcus melitensis*. Capsules have been described in freshly isolated strains, but if this be correct, motile and do not form are Gram negative.

The brucellæ are aerobic and do not grow under anaerobic conditions. The majority of stains of one species (*Br. abortus*), when freshly isolated, grow only in the presence of air containing a high proportion (optimum 10 per cent.) of CO_2 . After several generations, they may become adapted to grow in atmospheric air. Growth on artificial media, which takes place at body or air temperature, is always slow. Colonies may not become visible for 48 hours and the maximum size (3 mm.) may not be attained for 7 days. but these when this and ough

Maximum growth is obtained on liver extract agar. In broth, slight turbidity and deposit are slowly produced. Gelatin is not liquefied, indole is not produced and no sugar is fermented.

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The three species of the genus *Brucella* undergo S → R transformation more quickly than do most other bacteria. Even when

smooth organisms are agglutinated only by sera prepared with strains after intracutaneous cultivation for a variable period

the change has not been completed, the three species are antigenically indistinguishable. The occurrence of this type of transformation is of importance in another way. ✓ The power of the serum of a patient to agglutinate the

Br. melitensis is normally parasitic in goats, in which it causes abortion. When the acute stage of the disease has passed, the goat may continue to excrete the organism in large numbers in her milk and urine for a considerable time. The organism has occasionally been isolated from the milk of cows and sheep. Human beings may be infected directly from a patient or by material coming from a patient. ✓ The organism can enter the body through wounds and abrasions of the skin as has happened in a number of laboratory infections. The commonest method of

now usually

of infected goats. alta fever or
Mediterranean fever. It is characterised by headache, sweating, pains in the joints, orchitis, constipation, enlarged spleen and irregular pyrexia. ✓ It may persist for weeks or months. The fatality rate does not exceed 2 per cent.

Diagnosis may be made by isolating the organism from the blood or, less commonly, from the urine during life or from the spleen, lymphatic glands or gall bladder at post-mortem examination. ✓ All cultures should be incubated for several weeks as the

disease from animals and pasteurisation or boiling of milk.
 Br. melitensis is a frequent cause of abortion in cows and is

commonly present in the milk of animals which otherwise appear healthy. It may, in the British Islands, be present in 50 per cent. of samples of bulked, raw milk. In view of this, it is rather extraordinary that the number of human infections is so small and that those affected are rarely the chief milk consumers in a community, children and women. ✓ The human disease, abortus fever or the abortus variety of undulant fever, is a long continued pyrexia, often with remissions, which may be accompanied by joint pains, skin rashes and other symptoms. It is very rarely fatal. For purposes of diagnosis, a blood culture should always be made, preferably at the height of the pyrexial period (incubation being carried out in the presence of air containing 10 per cent. CO₂), but is commonly negative. A positive agglutination test, using the serum of the patient, usually suffices to confirm the diagnosis. It should be noted that when antisera, whether human or animal, are examined for their power to agglutinate brucella, marked zones are very liable to occur. So, in tubes containing high concentrations of serum, there may be no agglutination whereas, in later tubes in the series, in which the concentrations of serum are lower, marked agglutination occurs. ✓ The most satisfactory way of demonstrating the presence of *Br. abortus* in milk is by intraperitoneal or subcutaneous injection of a guinea-pig. The demonstration of agglutinins in the serum of the guinea-pig a few weeks after inoculating strongly suggests that the milk injected contained *Br. abortus*. ✓ Complete proof is afforded by killing the animal 2 months after inoculation and cultivating the organism from spleen and lymphatic glands. The milk of infected cows contains agglutinins, the presence of which may be demonstrated in the whey. It is very difficult to maintain a herd of milch cows free from *Br. abortus*. The only satisfactory method of prevention of human infections with the organism is pasteurisation of milk.

The usual host of *Br. suis* is the pig, but the organism may also cause disease in other animals, including the cow. The disease resulting from infection of human beings with this *Brucella* is similar to, but more serious than, abortus fever. Infection most commonly results from contact with a naturally infected animal or its carcass.

Agglutination :- Agglutinating to a high titre (1:1000) with anti-*Br. abortus* serum.

the change has not been completed, the three species are antigenically indistinguishable. The occurrence of this type of transformation is of importance in another way. The power of the serum of a patient to agglutinate one of the *Brucella* is of considerable value in establishing a diagnosis of infection by an organism of this genus, although not by a particular species. Since even partially rough strains are liable to non-specific agglutination by the sera of normal persons and, even more, of those suffering from pyrexia, only completely smooth strains must be used if

in which it causes disease has passed, the goat may continue to excrete the organism in large numbers in her milk and urine for a considerable time. The organism has occasionally been isolated from the milk of cows and sheep. Human beings may be infected directly from a patient or by material coming from a patient. The organism can enter the body through wounds and abrasions of the skin as has happened in a number of laboratory infections. The commonest method of infection, however, is by drinking the milk of infected goats.

The disease produced in man by *Br. melitensis* is now usually called undulant fever. It was formerly known as Malta fever or Mediterranean fever. It is characterised by headache, sweating, pains in the joints, orchitis, constipation, enlarged spleen and regular pyrexia. It may persist for weeks or months. The fatality rate does not exceed 2 per cent.

Diagnosis may be made by isolating the organism from the blood or, less commonly, from the urine during life or from the spleen, lymphatic glands or gall bladder at post-mortem examination. All cultures should be incubated for several weeks as the organism develops slowly.

completely smooth strain of
patient at a titre of 1 : 100

infection by some *Brucella*.

The preventive measures of importance are eradication of the disease from animals and pasteurisation or boiling of milk.

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commonly present in the milk of animals which otherwise appear healthy. It may, in the British Islands, be present in 50 per cent. of samples of bulked, raw milk. In view of this, it is rather extraordinary that the number of human infections is so small and that those affected are rarely the chief milk consumers in a community, children and women. ✓ The human disease, abortus fever or the abortus variety of undulant fever, is a long continued pyrexia, often with remissions, which may be accompanied by joint pains, skin rashes and other symptoms. It is very rarely fatal. For purposes of diagnosis, a blood culture should always be made, preferably at the height of the pyrexial period (incubation being carried out in the presence of air containing 10 per cent. CO₂), but is commonly negative. ✓ A positive agglutination test, using the serum of the patient, usually suffices to confirm the diagnosis. It should be noted that when antisera, whether human or animal, are examined for their power to agglutinate brucella, marked zones are very liable to occur. So, in tubes containing high concentrations of serum, there may be no agglutination whereas, in later tubes in the series, in which the concentrations of serum are lower, marked agglutination occurs. ✓ The most satisfactory way of demonstrating the presence of Br. abortus in milk is by intraperitoneal or subcutaneous injection of a guinea-pig. ✓ The demonstration of agglutinins in the serum of the guinea-pig a few weeks after inoculating strongly suggests that the milk injected contained Br. abortus. ✓ Complete proof is afforded by killing the animal 2 months after inoculation and cultivating the organism from spleen and lymphatic glands. The milk of infected cows contains agglutinins, the presence of which may be demonstrated in the whey. It is very difficult to maintain a herd of milch cows free from Br. abortus. The only satisfactory method of prevention of human infections with the organism is pasteurisation of milk.

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similar to, but more serious than, abortus fever. Infection most commonly results from contact with a naturally infected animal or its carcass.

Agglutination: Agglutinins to agglutination

CHAPTER XXXVIII

THE TUBERCLE BACILLUS

Mycobacterium tuberculosis var. *hominis*
Koch, 1884

Mycobacterium tuberculosis var. *bovis*
Th. Smith, 1896

Mycobacterium avium
Strauss and Gamaléia, 1891

There are three chief varieties of Tubercle Bacilli—the Human, the Bovine and the Avian. The first two are so similar that they are regarded merely as varieties of one species. The avian tubercle bacillus constitutes a distinct species. We will first consider the morphological and cultural characteristics of the three organisms separately, and will then deal with tuberculosis in general and the pathogenic properties of the three.

The human type of tubercle bacillus is a thin, straight or slightly curved rod measuring from 2μ to 5μ by 0.3μ . The bacilli are found in material taken from the body singly, in pairs usually arranged at an angle, or in clusters in which the individuals are commonly more or less parallel in arrangement. They may stain uniformly or may present a beaded appearance, due to the alternation of darkly staining portions with those which are either unstained or

the time is increased or heat is applied. When, however, the are stained, they very strongly resist decolorisation with alcohol or with mineral acids. These peculiarities—the difficulty in staining and the resistance against decolorising agents (the so-called acid-fast and alcohol-fast properties)—are most important points

in distinguishing them from the majority of other bacteria: the reason is probably the considerable amount of waxy and lipoidal substances present in their bodies. The most useful staining method is the Ziehl-Neelsen method, in which warmed carbol-fuchsin is the stain and the decoloriser either 20 per cent. sulphuric acid in water or 3 per cent. hydrochloric acid in alcohol. When the bacilli are stained by methyl violet they are found to be Gram positive. Under certain conditions young bacilli appear to be not acid-fast, and in tuberculous pus it may be impossible to

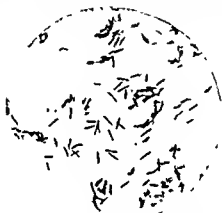


FIG 66.—*Myco. tuberculosis* FROM CULTURE ($\times 800$).

detect acid-fast bacilli, and yet the material may cause tuberculosis in animals when injected.

To obtain cultures of the tubercle bacillus special media and prolonged incubation are necessary. The slow growth prohibits the plating method commonly employed for the isolation of other bacteria, because the medium would become dry or would be overgrown by other organisms before the tubercle bacilli could develop. Cultures may be obtained by spreading material over the surface of special medium in a tube, provided no other organisms are present. To prevent drying of the medium, the tube may be sealed by treating the plug with melted paraffin wax. Much better results are, however, obtained if tubes or bottles

closed with screw caps are used. The methods used for obtaining cultures from sputum will be described later.

i) Coagulated egg media are the most useful for obtaining primary cultures. Egg medium may consist of eggs and water alone, the mixture being coagulated by heat, or may be modified by the addition of broth, glycerol, or other substances.

ii) For later cultures glycerol agar, glycerol broth, or glycerol potato may be used. While

primary cultures which, even in the case of the

glycerol egg media. ✓ Tubercle bacilli grow best aerobically at a temperature of 37° ; under anaerobic conditions the growth is always slight. ✓ Colonies of the human type bacilli begin to appear in from 10 to 14 days as minute greyish points. ✓ In subculture in from 3 to 4 weeks an extensive growth is generally obtained, which spreads over the greater portion of the surface of the medium as a dull, dry, wrinkled, or warty film. If plain egg medium is used, the colour is grey.



FIG 67 — CULTURE OF TUBERCLE BACILLUS (HUMAN VARIETY) ($\times 1$).

... the colour may be touched with have considerable

able cohesiveness, and an isolated fragment is difficult to break up in order to prepare a film. On glycerol broth, when growth is started by floating a thin piece of the culture film on the surface, a pellicle forms which spreads over the surface of the broth and for a little way above the surface on the sides of the flask. The pellicle increases in thickness and becomes wrinkled, closely resembling the growth on solid medium. The

organism grows in dispersed form in the medium of Dubos. In all essentials the bovine variety resembles the human very closely. It is, however, inclined to be rather thicker and shorter and also to show less variations both in size and appearance.

It has been noticed that on suitable culture media the human variety grows freely. To this peculiarity the term "eugonic" is applied. The bovine variety, on the other hand, is spoken of as "dysgonic" since, in earlier generations at least, the growth is never luxuriant. On egg medium in subculture it produces a thin, smooth, translucent film which is but slightly granular and is less dry than the human. This growth, when picked up, is found to be much less cohesive than the human. The growth of the bovine variety is not assisted by the presence of glycerol in the medium; indeed glycerol may have a considerable inhibitory effect in earlier generations. Glycerol, however, exercises less influence on cultures of long isolated strains of the bovine variety of bacillus.

The avian tubercle bacillus, while in morphology and staining characteristics it closely resembles the preceding, shows considerable differences in culture. It grows luxuriantly at 42° to 43° , a temperature sufficiently high to prevent any development of either the human or the bovine types. The growth is much more profuse and is definitely moist, in distinction to the dryness of cultures of the human variety. In colour it is inclined to be yellow or orange rather than greyish white.

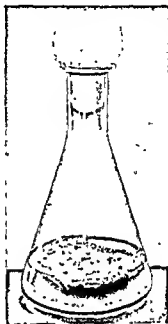


FIG 68—CULTURE OF TUBERCLE BACILLUS (HUMAN VARIETY) ON SURFACE OF GLYCEROL BROTH

closed with screw caps are used. The methods used for obtaining cultures from sputum will be described later.

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ii) For later cultures glycerol agar, glycerol broth, or glycerol potato may be used. While the addition of glycerol greatly promotes the

glycerol egg media. ✓ Tubercle bacilli grow best aerobically at a temperature of 37°; under anaerobic conditions the growth is always slight. ✓ Colonies of the human type bacilli begin to appear in from 10 to 14 days as minute greyish points. ✓ In subculture in from 3 to 4 weeks an extensive growth is generally obtained, which spreads over the greater portion of the surface of the medium as a dull, dry, wrinkled, or warty film. ✓ If the medium is new, the colour is grey,

colour may be touched with have considerable

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difficult to break up in order to prepare a film. On glycerol broth, when growth is started by floating a thin piece of the culture film on the surface, a pellicle forms which spreads over the surface of the broth and for a little way above the surface on the sides of the flask. The pellicle increases in thickness and becomes wrinkled, closely resembling the growth on solid medium. The



FIG. 67 — CULTURE OF TUBERCLE BACILLUS (HUMAN VARIETY) ($\times \frac{1}{2}$).

tuberculosis is fully considered in works on pathology it need not be detailed here. It is sufficient to recall the three types of cell found in the tubercle—the epithelioid cell, the giant cell and the lymphocyte. As the tubercle increases in size, necrosis of the central portion occurs, owing partly to the absence of blood supply but chiefly to the poisonous products of the bacilli. This process is spoken of as caseation, and the cheesy substance in the centre of the tubercle is called caseous material. Widely different pathological pictures result from the action of tubercle bacilli on different parts of the body, but the primary abnormality, the tubercle, is much the same everywhere. When the disease becomes advanced it is enclosed by fibrous tissue and the caseous mass may become calcified. In addition to the local result of invasion by tubercle bacilli (the production of tubercles) general effects are produced, especially pyrexia, sweating and wasting, as a result of the spreading by the circulation of some of the poisonous products of the bacilli. These effects are often accentuated by secondary infection with other organisms, which is very common in tuberculosis, particularly of the lung.

Usually tubercle bacilli may be found in tuberculous lesions, but the number is largest in acute and rapidly spreading disease, while in chronic tuberculosis very few bacilli may be found. When

moderately susceptible to the human variety of bacilli, the exact result of infection depending partly on the virulence and number of

Tubercle bacilli of all varieties show considerable resistance to unsuitable environments. In the dry state they can survive exposure to 100° for 20 minutes or more, but when moist a temperature of 60° kills in less than that time. In dried sputum the bacilli may remain viable for weeks or even months. They remain alive when exposed to 0° for several months, and still retain their pathogenic properties after an exposure to gastric juice for 6 hours. ✓ Their resistance to chemical agents, such as sodium hydrate or sulphuric acid, is shown by the chief methods used to free them from other bacteria in order to obtain pure



FIG 69 —TUBERCLE BACILLI IN SECTION OF LUNG ($\times 950$).

cultures. They are fairly rapidly killed by exposure to direct sunlight.

There is considerable ✓ overlap in the antigens of the varieties of the tubercle bacillus. By using the absorption of agglutinin technique, the avian variety can be distinguished from the human and the bovine, but these latter two cannot be distinguished from one another.

Tuberculosis in man is due chiefly to the action of the human variety of tubercle bacillus, to a lesser extent to the bovine variety and very rarely to the avian. ✓ The fundamental lesion in the bodies of man or animals is the tubercle. Since the pathology of

tuberculosis is fully considered in works on pathology it need not be detailed here. It is sufficient to recall the three types of cell found in the tubercle—the epithelioid cell, the giant cell and the lymphocyte. As the tubercle increases in size, necrosis of the central portion occurs, owing partly to the absence of blood supply but chiefly to the poisonous products of the bacilli. This process is spoken of as caseation, and the cheesy substance in the centre of the tubercle is called caseous material. Widely different pathological pictures result from the action of tubercle bacilli on different tubercles.

retrograde so walled off, and at a still later stage the caseous mass may become calcified. In addition to the local result of invasion by tubercle bacilli (the production of tubercles) general effects are produced, especially pyrexia, sweating and wasting, as a result of the spreading by the circulation of some of the poisonous products of the bacilli. These effects are often accentuated by secondary infection with other organisms, which is very common in tuberculosis, particularly of the lung.

Usually tubercle bacilli may be found in tuberculous lesions, but the number is largest in acute and rapidly spreading disease, while in chronic tuberculosis very few bacilli may be found. When the disease is an "old" one, that is where one or more foci are

moderately susceptible to the human variety of bacilli, the exact result of infection depending partly on the virulence and number of

variety are rarely encountered. In the case of young children, however, the condition is different, for these possess but little

i The great
the human

In the case of other animals the bovine variety is much the more virulent. The rabbit, injected intravenously with 0.01 mg. of a culture of the bovine variety, dies in about a month with widespread progressive tuberculosis. A similar injection of the human variety is usually not fatal and causes only slight lesions, chiefly in the lungs and kidneys. Subcutaneous injection of the human variety in the rabbit fails to kill and produces a local lesion and small lesions in the lungs and kidneys; the bovine, on the other hand, causes death in 2 to 3 months with generalised tuberculosis. This difference between the two organisms in their pathogenicity for animals, particularly for the rabbit, is one of the characteristics which enable them to be distinguished. ✓ The guinea-pig is susceptible to the human variety, but distinctly more so to the bovine. When injected subcutaneously, death results in about 3 months with the bovine variety and rather later with the human. In both cases there is to be found a local caseous lesion, involvement of the various groups of lymphatic glands draining the area, and usually of the spleen, liver, and lungs. Inoculation of a guinea-pig is one of the methods used for isolating tubercle bacilli and also for their identification in suspected material.

The tubercle bacillus is a strictly pathogenic organism and, apart from laboratory cultures, appears to be incapable of acting as a saprophyte. Infection is, therefore, from case to case either directly or indirectly. ✓ The bacilli in human disease are excreted chiefly from the respiratory, intestinal, and urinary tracts, especially the first. → The bacilli may be carried directly from a case to a fresh victim in droplets of sputum or indirectly in dust composed of dried sputum containing bacilli. ✓ The prolonged vitality of the bacilli in the dry condition and the common habit of promiscuous expectoration are the two chief factors which make tuberculosis the scourge it is to-day. In the case of the human variety

the bacilli passing either from the bladder or intestine are much less commonly the cause of fresh cases. In the case of animals, in which infection may occur as a result of contamination of pastures, the intestinal route is of greater importance than it is in man. The chief agency by which bovine tubercle bacilli are conveyed to man is the milk of infected cows. In these animals tuberculosis of the udder is a fairly common condition, but even when the udder has no trace of the disease the bacilli from other parts of the body may be excreted in the milk. Under dirty conditions cow's milk contains a considerable amount of faeces and, when



FIG. 70 —TUBERCLE BACILLI IN DEPOSIT
FROM URINE ($\times 950$)

tuberculosis of the intestine exists, the milk may contain a large number of the bacilli. The flesh of tuberculous animals can be responsible for only a small number of cases of tuberculosis in man in countries where raw meat is not a common article of diet. Further, it is rare for the flesh (i.e. muscle) of animals to become tuberculous, even when the glands are extensively involved.

The route of infection in man may be either by inhalation, by ingestion, or by the skin. That inhalation is the commonest of these there can be little doubt, but as to the exact method by which the bacilli reach the lung, the usual site of the disease, there is still little agreement. In some cases they are carried in the air

to the deeper parts of the respiratory tract. ✓ Another method appears to be by penetration of the epithelial lining of the upper part of the tract and thence, by the lymph stream, to the mediastinal glands and the lung itself. ✓ The tonsils may be one of the most important portals, and as a result may show evidence of tuberculosis, or more commonly may be unaffected. Bacilli ingested either in dust or in food may cause tuberculosis of the intestine, but there is no doubt that they are capable of penetration.

✓ Tuberculosis due to ingestion, which occurs most commonly in children, may be due to the bovine type of bacillus. The most common source of these bacilli is the uncooked milk of tuberculous cows. Skin infection may occur in butchers, veterinary surgeons, and others who handle infected material. This form of the disease tends to remain localised in the skin and is very chronic. ✓ Lupus vulgaris is another type of tuberculous skin lesion which occurs most commonly about the face. The mode of infection in these cases is uncertain.

✓ The widespread distribution of tuberculous lesions throughout the body in advanced cases can be explained only by the transference of the bacilli by the blood stream, but the claims of certain workers to have isolated tubercle bacilli from the blood in a large proportion of cases is not generally accepted.

Tubercle bacilli in the body cause injury owing to the various toxic substances produced by their growth. ✓ Although filtrates of broth cultures may produce, on injection, toxic effects such as fever and an acute inflammatory reaction, it is doubtful if the bacilli form any true extracellular toxin. ✓ The toxins produced, of which there are probably several distinct varieties, are entirely endotoxins. ✓ Dead bacilli in the animal's body, are capable of producing local

importance when animal inoculation is used for the identification of living tubercle bacilli unless the dose is large, caseation does not occur, and is responsible for the

The problem of immunity in tuberculosis is one of great difficulty and complexity. The disease tends to be a chronic one and, even in its most acute forms, is much more prolonged than the majority of bacterial diseases. Tubercle bacilli are so prevalent that few adults who have lived in urban communities can have avoided all contact with them. Some of those exposed to infection escape completely and some develop progressive disease but, in the majority, the organisms establish a focus in the body which,
ed, leaving
tuberculin
detectable
by X-ray examination. We have little evidence to show that cure is due to specific antibacterial or antitoxic substances produced by the body in response to the stimulus of the bacilli. Rather, cure seems to depend on the general health and resistance of the body and to be promoted, to a great extent, by such general factors as nutrition and environment. In the human body, during the disease, certain types of antibodies may be found—agglutinins, precipitins, opsonins and complement fixing substances—but their exact significance cannot, as yet, be claimed to be thoroughly known. Complement fixation, for example, may be strong in those showing no clinical trace of tuberculosis and weak or absent in those in a very advanced stage of the disease. In tuberculosis instead of the patient tolerating well considerable amounts of the products of growth of the tubercle bacillus, as one would expect, there is developed a very great sensitivity to them. So we find that a patient with tuberculosis may be rendered very gravely ill, or may even die, as a result of an injection of an amount of one of these products, Tuberculin, which would be almost without effect on a healthy individual. Hypersensitivity to this product can be more safely demonstrated by the various local tuberculin reactions considered later

It is very difficult to write dogmatically about the relationship of hypersensitivity to tuberculin and immunity in tuberculosis. It appears simplest to regard hypersensitivity and immunity as developing quite independently of one another, but both as the

result of an actual infection with the tubercle bacillus. Infection may lead to the development of a latent focus.

from nor immune to tuberculosis. A person who gives a positive tuberculin reaction, however, may, in the past, have developed sufficient immunity to have overcome a now extinct infection and this immunity may have persisted or, on the other hand, he may be suffering from an actual infection, whether latent or actively progressive. There is a considerable body of evidence which suggests that the person most likely to escape tuberculosis, despite frequent exposures to infection, is one whose tuberculin reaction is positive but in whose lungs X-ray examination fails to reveal the presence of an active focus.

✓ Tuberculin was the name given by Koch to a concentrated and filtered glycerol broth culture of tubercle bacilli. This material, sometimes called Old Tuberculin or T., contains all the substances of the culture medium, including those formed from the medium by the bacteria, and the disintegration products of the bacteria themselves. Purified Protein Derivative (P.P.D.) is prepared from a culture of the tubercle bacillus grown on a synthetic medium. It is a relatively pure preparation of the active principle of tuberculin. Batches of tuberculin and of P.P.D. may show some variations in potency, and so, before being issued for use, every batch must be standardised. ✓ The standard is a particular sample of Old Tuberculin kept in one of the Standards Laboratories. A new batch of tuberculin is standardised by comparing the reactions produced by the intradermal injection of various dilutions of it and of standard tuberculin in infected guinea-pigs.

The amount of diluted tuberculin inoculated intradermally in the Mantoux test is usually 0.1 ml., and differences in dosage are secured, not by varying the volume injected, but by using different dilutions of tuberculin. So we may inject, in successive tests, 0.1 ml. of 1 : 10,000, 0.1 ml. of 1 : 1,000 and 0.1 ml. of 1 : 100 Old Tuberculin or corresponding amounts of solutions of P.P.D. Confusion is very liable to arise as a result of the use of a variety

of methods of denoting the strength of the material injected. These include Tuberculin Units (T.U.), milligrams of Old Tuberculin (T) or milligrams of dry P.P.D. Since errors in dosage may have very serious consequences, the following table of equivalents is given. When we use the dilution method of expression, we should state the volume of this dilution injected, e.g. 0.1 ml. of 1:1000 tuberculin but, since 0.1 ml. is almost universally the volume used, this is often omitted. When the dose is expressed in Tuberculin Units or in milligrams, it is of no importance whether the volume is 0.1 ml. or 0.2 ml., provided that the required number of units or weight of substance is contained in the volume injected.

0.1 ml. of 1:10,000 tuberculin is equivalent to

1 T U
0.01 mg. T
0.00002 mg P P D

0.1 ml. of 1:1,000 tuberculin is equivalent to

10 T U
0.1 mg T
0.0002 mg P P D

0.1 ml. of 1:100 tuberculin is equivalent to

100 T U
1 mg T.
0.002 mg P P D

Tuberculin was designed for the treatment of tuberculosis. It is not a cure for the disease, although its proper use may assist other therapeutic and, more important, hygienic measures, especially in localised tuberculosis of kidneys, glands, bones, and joints. The hypersensitivity of the tuberculous patient to the bacilli or their products may be shown by the reactions which occur as a consequence of the administration of a small dose of tuberculin. These may be either local (e.g. skin tests) or general (e.g. fever and general reactions would appear to be due to the action of the injected tuberculin on the tubercles, in which there follows hyperæmia, softening and liberation of poisonous substances into the circulation). The original aim of tuberculin therapeutics was to provoke this focal reaction, for following it there is an active stimulation of the surrounding cells to proliferate and this may lead to encapsulation of the diseased focus. The focal reaction

must, however, be kept quite slight, as otherwise there may be caused an acute exacerbation of the disease with rapid spread.

Several diagnostic tests have, as their basis, the sensitivity to tuberculin which develops in those infected with the tubercle bacillus. The original one was performed by the subcutaneous injection of tuberculin, but this is too dangerous to use for man. 10 ml. of tuberculin injected into a new-born infant is practically without effect, but a much smaller amount (0.01 to 0.0001 ml) in a tuberculous subject may cause very severe local, focal and general reactions. The test now most commonly used in human beings is that of Mantoux, in which 0.1 ml. of a 1 : 10,000 dilution of tuberculin is injected intradermally. In the positive, redness and œdema develop within a few hours and reach a maximum next day. Vesication may occasionally occur. If there is no reaction the test should be repeated, using 0.1 ml. of a 1 : 1000 dilution and, if this is negative, 0.1 ml. of a 1 : 100 dilution. Other methods of testing a person for hypersensitivity to tuberculin are by rubbing into a small area of skin an ointment containing tuberculin (Moro test) or by sticking to the skin a plaster incorporating tuberculin (Vollmer patch test). In either case a red reaction of the skin indicates hypersensitivity.

The various tuberculin tests, as diagnostic methods, all suffer from the same disadvantage—they give positive results not only in persons actually suffering from tuberculosis but also in persons who have formerly suffered from tuberculosis. The condition of hypersensitivity persists long after the disease has become cured. A negative result, however, especially if given by the Mantoux method with a 1 : 100 dilution, indicates with a high degree of probability that the person has never been infected by the tubercle bacillus. The tests are, therefore, chiefly of value in children. The intradermal test is used in testing cattle. If a milk cow gives a negative result in a properly carried out test, it is almost certain that she is not suffering from tuberculosis.

Neither dead vaccines of tubercle bacilli nor tuberculin give rise to immunity against tuberculosis. A living vaccine, consisting of a strain of bovine tubercle bacilli (Bacille Calmette-Guérin,

B.C.G.) deprived of virulence by prolonged culture on bile medium, has been extensively employed in France, the Scandinavian countries and elsewhere, to produce active immunity against the disease. It is best injected intradermally in two or three situations. Local nodules are produced which only rarely break down and discharge. There is no general dissemination of the organism. B.C.G. is of greatest value in protecting infants born to tuberculous mothers and such persons as nurses whose occupation renders them especially liable to infection. As a result of inoculation with B.C.G. the tuberculin reaction is converted from negative to positive and it is the general opinion of those best qualified to judge that this vaccine is of real value in the prophylaxis of tuberculosis. Since the bacilli must be alive when introduced into the skin, a batch has a useful life of only 10 days or so after its preparation. It is possible that the variety of tubercle bacilli isolated by Wells from voles (*Myco. tuberculosis* var. *muris*, or *Myco. muris*) may ultimately replace B.C.G. as a living vaccine since it is almost devoid of virulence for human beings.

Since the acid- and alcohol-fastness of the tubercle bacillus is the characteristic on which we chiefly rely for its identification, a consideration of the other bacteria with similar staining properties is necessary. The most important of these acid-fast bacilli is *Myco. lepræ*, which will be considered separately. There are a number of other organisms of which *Myco. phlei* (Moeller's grass bacillus) and *Myco. butyr* (the butter bacillus of Rabinowitsch) may be taken as types. These bacilli resist decolorisation with acids, but are easily distinguished from the tubercle bacillus by their rapid growth, even at air temperature, on ordinary media. They are practically non-pathogenic for animals, although a large injection may cause a slight local lesion. Another acid-fast organism is *Myco. paratuberculosis* (John's bacillus) which is found in enormous numbers in the thickened mucous membrane of the small intestine of cattle suffering from chronic paratuber-

genitals of both sexes. It is rather shorter than the tubercle bacillus and is described as being acid-, but not alcohol-fast; not much reliance can be placed on these characteristics, since different types vary greatly in these respects, and some resist decolorisation with alcohol very strongly.

The finding of acid- and alcohol-fast bacilli of typical shape in material coming from the interior of the body—sputum, cerebro-spinal fluid or pus from an unopened cold abscess—is, for all practical purposes, conclusive evidence of tuberculosis. Fluids such as cerebro-spinal fluid, pleural fluid, or urine, should be centrifuged and films made from the deposit. In the case of cerebro-spinal fluid, films should also be prepared from the fine web-like clot which forms as, in it, many of the tubercle bacilli present are likely to be found. The nature of the cells present in the

care is required in the microscopic examination of urine, owing to the possibility of smegma bacilli being present. If, however, acid- and alcohol-fast bacilli of typical morphology are found in a catheter specimen of urine in which pus is present and no growth is obtained in 48 hours on culture, they are, almost certainly, tubercle bacilli. It is doubtful if it is ever correct to report tubercle bacilli as present in feces without animal inoculation.

Where any material is being examined directly for the presence of tubercle bacilli, it should be borne in mind that it is practically impossible either to over-stain or over-decolorise in the Ziehl-Neelsen method.

Since sputum is the material most frequently examined for the presence of tubercle bacilli, the various methods employed will be considered in some detail. Four methods are available: (1) direct microscopic examination; (2) microscopic examination after concentration; (3) culture; (4) animal inoculation.

For direct examination, smears are made from selected particles of purulent material. Care must be taken to distinguish these from

particles of food. The smears are stained by the Ziehl-Neelsen method.

When direct smears fail to reveal tubercle bacilli, concentration is sometimes used. Add to some sputum in a test tube an equal volume of 0.2 per cent. NaOH, shake, heat for 10 minutes at 100°, and centrifuge. Films are prepared from the deposit. In staining these, the treatment with carbol fuchsin should be applied for 20 minutes, as the alkali tends to cause weak staining. We have not found concentration methods superior to the direct examination of films prepared from selected portions of sputum.

When microscopic examination fails, either culture or animal inoculation or, preferably, both may be attempted.

Culture, if properly done, can be just as reliable as animal inoculation and may give a positive result earlier. Sputum should not be spread directly on media because the contaminants present in it grow rapidly and so destroy the cultures. The contaminants must, therefore, be killed before cultures are made and, at the same time, tubercle bacilli must be set free from the necrotic material. Both these are accomplished by adding to some sputum in a sterile test tube an equal volume of 5 per cent KOH and incubating at 37° for 45 minutes. The mucilaginous suspension obtained is spread by means of a Pasteur pipette on at least two
 5 per cent.
 : better is
 medium is

prevented by using slopes in tubes or bottles closed with screw caps. The cultures should be examined on the third day for contamination. If the latter is heavy, the original sputum or a fresh specimen should be treated with an equal volume of 3.4 per cent. (by volume) H_2SO_4 in distilled water at 37° for half an hour. The mixture is then diluted with 10 volumes of sterile saline and centrifuged and the deposit spread on the media as before. Alternatively, the H_2SO_4 treatment may be used at the outset instead of KOH. Cultures should be examined at least once a week with a hand lens ($\times 6$). The colonies, which may be only pin-point in size, appear in from 2 to 4 weeks. Smears should be

made from suspected colonies, and these should be spread over the slope as soon as they are seen.

- ✓ Sputum intended for animal inoculation should be treated as for culture since, if injected without treatment, other bacteria present may cause acute infection and death of the animal before tuberculosis has developed. Either method (KOH or H_2SO_4) may be used, and the mixture, after treatment, must be neutralised (with H_2SO_4 or KOH) to litmus or phenol red before injection.
- ✓ A total volume of 4 ml. may be injected intramuscularly into the thigh of a guinea-pig's hind leg. Guinea-pigs used for this purpose should weigh 300 g. or more.

In positive cases, the inguinal lymphatic glands become palpable in about 3 weeks. The animal should be killed at the end of 6 weeks.

The post-mortem findings in a positive case are as follows: a local caseous or purulent lesion at the site of inoculation; enlarged superficial and deep inguinal glands on the affected side which may be as large as a cherry and may be solid or caseous; the superficial inguinal glands on the opposite side may be enlarged, the lymphatic glands at the bifurcation of the abdominal aorta, the preaortic glands at the level of the kidneys and the portal gland, above and behind the pylorus, may be enlarged and even caseous, the spleen, which normally measures 2×1 cm., is enlarged, sometimes to as much as 8×5 cm., dark red in colour and shows a variable number of irregular, white or cream necrotic areas varying in size from 0.5 mm. to 2 cm. in diameter; the liver, which is enlarged and pale, usually contains only a few irregular cream or yellow areas of similar size, the lungs may be studded with pinhead or larger irregular foci, cream in colour with green margins.

That the various lesions are tuberculous should be confirmed by smears stained by the Ziehl-Neelsen method. Errors may arise from the existence in the animal of pseudo-tuberculosis, a disease caused by a Gram-negative, bipolar bacillus, *Pasteurella pseudo-tuberculosis*.

The above methods of examination may be used for any other

material, such as pus, but unless there is gross contamination, treatment with 5 per cent. KOH need only be for 30 minutes at 37°. ✓ Where sputum is not produced or where it is difficult to collect, results at least as good as those given by sputum may be obtained by culture or animal inoculation of the fluid obtained, on waking in the morning, by gastric lavage. The fluid should be treated by the KOH method.

Saprophytic acid-fast bacilli are frequently found on corks and rubber bungs so that, if either of these are used for sputum containers, they should be thoroughly washed and sterilised before use. Saprophytic acid-fast bacilli do not cause lesions in a guinea-pig resembling those of tuberculosis and do not kill.

While the finding of tubercle bacilli is the only certain method of making a diagnosis, it may sometimes fail even with the greatest care. Then the patient's hypersensitivity to tuberculin may be tested, but it must be borne in mind that a positive reaction may be due to a healed focus and that a negative result may be obtained in very advanced cases.

The examination of the serum of a patient for antibodies to the tubercle bacillus by agglutination, precipitation or complement fixation methods is not of great diagnostic value. ✓ A positive result may be obtained in the apparently healthy and a negative one in persons suffering from advanced tuberculosis

The only chemotherapeutic substance which is effective against the tubercle bacillus and at the same time not excessively toxic is streptomycin. Its most successful application is in the case of tuberculous meningitis in which it is injected into the cerebro-spinal space. By the use of this substance, a considerable number of patients have been cured of this hitherto invariably fatal disease.

CHAPTER XXXIX

Mycobacterium lepræ Hansen, 1879

Myco. lepræ is an organism which, in many respects, resembles the tubercle bacillus. It is of about the same size, but is inclined to be slightly more slender and is less frequently curved. Its ends are commonly pointed, but occasionally slight clubbing is observed. It may stain uniformly, but beaded forms are of frequent occurrence. It stains with fair ease by any of the ordinary aniline dyes and is Gram positive. When stained like the tubercle bacillus, it resists strongly the decolorising effect of mineral acids and so the Ziehl-Neelsen method is commonly used for its demonstration. Generally, although not invariably, it is more

leprosy
pecially
lepra
ularly
of the

anæsthetic form of the disease. They have often been demonstrated in the blood and in the internal organs, particularly the spleen, liver and kidney, and are almost universally present in the nasal mucous membrane and nasal secretions of lepers.

The present position with regard to the cultivation of the organism is most unsatisfactory. A large variety of cultural methods have been used and a considerable number of distinct organisms have been cultivated from leprosy lesions. Each has been claimed as being the leprosy bacillus, and while the evidence brought forward in support of the claims of some of these must be treated with respect, none has been definitely proved to be the causal organism of leprosy.

ment of granulation tissue, which occurs either in the form of nodules or as a diffuse infiltration of the skin or mucous membrane. This form is most frequent in the nose and the face, but the hand and the inter

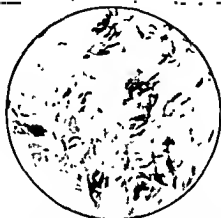


FIG 71.—*Mycobacterium leprae* IN FILM FROM NOSE ($\times 950$)

granulation tissue contains large numbers of mononuclear leucocytes, but its most remarkable feature is the occurrence of "lepra cells", large cells with clear nuclei. Bacilli may be seen within these cells. In the nose, the effects of the disease are less marked and the effects are mainly due to cicatrisation. Anæsthesia and paralysis are the striking features of this form of the disease, and later trophic disturbances help to produce great disfigurement.

tion rather than to leprosy itself. In no other condition is so much disfigurement and crippling produced with so little evident

impairment of the general health. The leprosy bacillus is capable of producing very marked local effects but is only slightly toxic.

The results of animal experiments have been disappointing, since the majority of animals appear to be very resistant to *Myco. lepræ*. Successful inoculations, with the production of nodules containing acid-fast bacilli, have been recorded in certain monkeys and hamsters, but the lesions have never been progressive. The finding of acid-fast bacilli locally in animals inoculated with leprosy material must be received with caution, since so many lepers are also tuberculous. The small percentage of successful animal infections and the insignificant lesions produced when material containing true leprosy bacilli has been used, render difficult the identification of organisms found in cultures. It must also be realised that many of the non-pathogenic, acid-fast bacilli, when injected in large numbers, may give rise to nodule production, the bacilli occurring in the nodule.

As regards the spread of the disease, it is certain that its infectivity is very slight. Both intimate and prolonged contact are necessary for infection. The exact route is unknown, but the fact that the nasal secretions commonly contain large numbers of bacilli is suggestive.

The finding of enormous numbers of acid-fast bacilli in a human lesion and the absence of effect when a guinea-pig is inoculated with such material is usually sufficient to establish the diagnosis. It should be noted that many of the ulcers may be trophic and may contain no bacilli.

CHAPTER XL

Malleomyces mallei (*Pfeifferella mallei*) Löffler, 1886

Malleomyces mallei, the causative organism of glanders, is a straight or slightly curved, round-ended bacillus, measuring from 2μ to 5μ by from 0.5μ to 0.7μ . In cultures both long filaments (which may be branched) and almost coccid forms are to be seen. The bacillus is non-motile, has no capsule, and does not form spores. It stains feebly with watery solutions of the basic aniline dyes, but more deeply if the stain is alkaline (Löffler's methylene blue) or contains a mordant (carbol fuchsin). One of its chief characteristics is the irregularity with which different parts of the bacillus take the stain. It is Gram negative and is not acid-fast.

Mall. mallei is aerobic and, under anaerobic conditions, growth is very poor. It grows moderately well but rather slowly on ordinary media. Growth is facilitated by the addition of glycerol to the medium. On potato a slimy, yellow, transparent layer appears which resembles a smear of honey, later the colour darkens until in about a week it is reddish-brown or chocolate. *Mall. mallei* ferments glucose slowly; no other carbohydrate is affected. It either does not liquefy gelatin or liquefies it very slowly. Indole is not produced.

The resistance of the organism to heat and antiseptics is not striking. It can, however, survive for 1 or 2 months in water, an important practical point in connection with horses' drinking-troughs.

Glanders is a disease essentially of the horse family to which many other animals, including man, are more or less susceptible. The chief feature of the disease is the production, in tissues infected with the bacillus, of the characteristic nodules which are composed of leucocytes (chiefly polymorphonuclear), epithelioid cells and

connective tissue. Pus is not usually produced, but the central cells generally degenerate and disintegrate and the nodules become soft and break down, forming ulcers. In the more chronic nodules the connective tissue development is exaggerated and the leucocytic invasion is less marked. Bacilli are fairly plentiful in the acute lesions, mostly extracellular, but a few lie within the leucocytes. It may be impossible to find any in old chronic cases.

The horse may suffer from either the acute form of glands or from the chronic form, known as farcy. In both, nodules which break down producing ulcers occur either in the nasal mucous membrane or in the skin.

In man, glands may be either acute or chronic. The primary lesion is generally in the skin, more rarely in the mucous membrane of the nose, mouth or eye. There is a local, nodular swelling and inflammation of the lymphatic vessels and also a general skin eruption, at first papular, later pustular. The disease is almost invariably fatal.

The disease is spread from horse to horse chiefly by the infective nasal secretion and by the discharge from ulcers. Infection occurs through the skin or mucous membrane, by inhalation, or by ingestion dealing diseases known.

Mall. mallei is pathogenic for the usual laboratory animals, a fatal condition being produced. In a male guinea-pig, following intraperitoneal injection, a purulent orchitis is set up which is almost diagnostic. This is known as the Straus reaction.

During the course of the disease but little immunity is established and an animal, which has suffered from a chronic form of the disease, may die within a few days from an acute exacerbation.

Other antibodies may be found in infected animals, and may be used for diagnosis, but these do not always appear. Just as in tuberculosis, the bacilli are present and

this is made use of in a diagnostic test for the disease in which a product known as mallein, prepared in a manner almost identical with that of old tuberculin, is employed.

The microscopical characteristics of the bacillus in young lesions may be almost sufficient for diagnostic purposes but no bacilli may be found in old lesions. If an unbroken nodule is available, direct culture should be attempted on glycerol agar, potato and serum, but, where other bacteria are present, one often fails to obtain a culture by plating.

Intraperitoneal injection of a male guinea-pig is a valuable method both for diagnosis and for the isolation of the bacilli for culture, since they are commonly found in a pure condition in the tunica vaginalis of the testicle. Certain indirect methods of diagnosis are also available, the most reliable of which are agglutination and complement fixation, using the serum of the affected animal. A simple and reliable test for the disease in horses is the subcutaneous or intradermal injection of mallein. During the course of the disease hypersensitivity to mallein develops and therefore the occurrence of a negative reaction in the

of the reactions produced, it should not be employed for diagnosis in suspected cases of the disease in man.

Melioidosis, a rare tropical disease resembling glanders and affecting man, guinea-pig, rabbit, dog, cat and rat, is due to *Malleomyces pseudomallei* (*Pfeifferella whittmori*). This bacillus is motile, grows luxuriantly on agar, ferments lactose, glucose and other carbohydrates, rapidly liquefies gelatin and does not produce indole Antigenically it is almost identical with *Mall. mallei*.

CHAPTER XLI

SPIROCHÆTES

or may be imaginary as is the case with the genera containing pathogenic species. The classification and nomenclature of this order are full of difficulties. We propose to follow Bergey's Manual in using Borrelia, Treponema and Leptospira as the names of the three genera. While it is quite wrong to apply to these organisms the generic name Spirochæta, there is no objection to calling them all spirochætes.

THE RELAPSING FEVERS:-

In various parts of the world a type of fever, which is characterised by the alternation of periods of pyrexia with periods in which the temperature is normal, is of fairly common occurrence. In these fevers, spirochætes are present in the blood stream during the pyrexial periods. Although these spirochætes are all very similar in general appearances and effects, they are to be regarded as belonging to distinct species. During the relapse the spirochætes return to the peripheral blood.

Borrelia obermeieri (*Treponema recurrentis*)

Obermeier, 1873 / Schöberl, 1973.

length is constant in length, varying from 10.4 μ . Its forms. They are fairly regular and well marked oval, during life do not rigidly hold their shape, the whole organism bending and straightening itself. It exhibits three forms of movement → progression in either direction, a rotating or corkscrew motion, and a corkscrew motion → (pathogenic humanus) No flagella

and alternate bending and straightening. When a drop of infected blood is examined fresh the parasites are seen to move actively, pushing the blood cells from their path. During the height of the fever the number of spirochætes in the circulation may be enormous, several being visible in each microscopic field. The parasite stains faintly with watery solutions of the aniline dyes, but much

ascitic fluid
the chorio-

allantoic membrane of the developing chick embryo.



FIG 72.—*Borrelia obermeieri* IN BLOOD FILM

In man, after an incubation period, there is a rapid rise of temperature and a pyrexial period of from 5 to 7 days followed by crisis. After about a week another period of fever occurs which as suddenly gives place to normal temperature. In all, three or four paroxysms may occur, each being of less severity and shorter duration than the preceding. The mortality is low and the post-mortem findings are not noteworthy; enlargement of the spleen and liver with gastric catarrh being of most common occurrence. The spirochæte appears in the blood in cases of the human disease shortly before the temperature begins to rise, and the number increases until fever is at its height. They remain

crisis - the turning point of a disease for good or evil as it changes immediately in the latter, in the course of an

numerous throughout the greater part of the period of pyrexia, but shortly before the crisis they disappear, and none can be found until the beginning of the relapse.

The nature of the immunity in this disease is of interest. At the crisis, in the segment by the

finding that, although spirochaetes can live for weeks in blood removed early in the pyrexial period, they are quickly killed by blood taken at the time of crisis. Despite the presence of antibodies a few spirochaetes

have effected a change in serological type. Against them the patient develops new antibodies which cause a crisis and the process is the spirochaetes. In each case the a later relapse

sign.

This variety of relapsing fever is of most common occurrence in Europe. The organism is conveyed by the body louse and, less frequently, by the bed bug. Infection may be caused by the feeding of the insect, but more commonly by rubbing into the wound the coelomic fluid of the crushed louse, in which spirochaetes are present in large numbers. (See also *relapsing fever*.)

Very similar spirochaetes have been found to be the cause of relapsing fevers in other countries—*Borrelia novyi* in America, *Borrelia carteri* in India and *Borrelia berbera* in Algiers. These organisms present no marked peculiarities sufficient to distinguish them from *Borrelia obermeieri* or from one another.

African relapsing fever or Tick Fever is caused by *Borrelia duttoni*. This disease is characterised by short periods of pyrexia (2 or 3 days), many relapses, and very low mortality. It is conveyed by a tick, more usually by its excretions than by biting, and the chief point of interest is that a tick may remain infective for

periods of many months after a meal from an infected host.

after a meal, it is probable that certain minute chromatin granules, which have been described as making their way through the tissues of the tick and appearing within the ova, are a stage in the development of the organism.

The borreliae of the relapsing fevers are pathogenic for monkeys, mice and rats, but there is no evidence that these animals suffer from naturally contracted infections.

Until the introduction of penicillin, which is effective in the treatment of the majority of spirochætal infections, salvarsan and its derivatives were probably the most valuable drugs in the treatment of the relapsing fevers.

SYPHILIS AND YAWS

Treponema pallidum
Schaudinn and Hofmann, 1905

Neurotropic.
Dermotropic.

This organism, which is usually about 8μ in length, may measure from 3μ to 18μ , and is extremely slender, rarely exceeding 0.25μ in thickness. It is characterised by sharp, deep, regular spirals, the number of which depends on the length of the individual. The size of each spiral is fairly constant, a distance of about 1μ separating the crest of one from that of the next, while the depth of the curve is about 1μ . The parasite tapers to each end and terminates in a fine thread which is not usually regarded as a flagellum. Until the introduction of the electron microscope, it was accepted that an important characteristic of genera of the order *Spirochaetales* was the absence of flagella. In some photographs of *Trep pallidum* obtained with this instrument, however, long, thin processes, indistinguishable from flagella, are clearly visible. These occur in tufts of two to four, not at the end of the spirochæte, but along its length. During life *Trep pallidum* is fairly motile. The regularity of the spiral is unchanged during r.

actively motile, showing progression in either direction, rapid rotation around its long axis, expansion and contraction of its spirals, and also slight flexion of the whole body.

It is alleged that this *Treponema* has been cultivated by Noguchi's technique under anaerobic conditions in a semi-solid mixture of agar and hydrocele fluid containing a piece of sterile rabbit's tissue, either kidney or testicle. There is considerable doubt as to whether the organisms cultivated in any of the very few successes which have been recorded were actually *Trep pallidum*. *The cultivated organisms do not retain its virulence on subculture.*



FIG. 73 —*Treponema pallidum* FROM TONSIL WARTHIN'S STAIN (X 1200)

The organism's powers of resistance are very slight, drying or moderate heating (55°) being rapidly fatal, but cold is well borne. The organism stains only with great difficulty (from which the ordinary aniline Giemsa, Fontana, or Levaditi) or the Indian ink method may be used, but in the case of fresh material the best results are obtained by examining the living organism unstained On account of its low refractivity, the usual method of illumination does not reveal it, and indirect lighting, by the use of the dark field condenser, must be employed. It is unnecessary to describe here the many clinical manifesta-

tions of sypilis. The *Treponema pallidum* has been found in every lesion characteristic of the disease. It occurs in greatest numbers in the primary lesion, the chancre, and its identification in that situation is the earliest and simplest method of diagnosing the disease. The treponemata may usually be found without difficulty in secondary lesions, and Warthin has shown that in gumata and other tertiary lesions prolonged search may reveal large numbers

The disease is most commonly acquired in sexual intercourse, but non-venereal infections also occur. It is unlikely that the

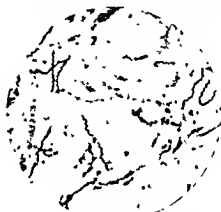


FIG 74 —*Treponema pallidum* IN LYMPH GLAND WARTHIN'S STAIN (X 800)

organisms are present, the unbroken skin or mucous membrane, terial
The
sexual
intercourse by the use of soap and water, with or without an antiseptic, shortly after connection, is undoubted. A most important type of innocent infection is that seen in congenital sypilis, the disease being acquired in utero from an infected mother, very rarely, if ever, from the father in the absence of sypilis in the mother.

The majority of animals are completely refractory to infection

by *Trep. pallidum*. A disease similar to human syphilis, but progressing to the secondary stage only, is produced in the chimpanzee by inoculation. Keratitis, iritis and orchitis may be produced in rabbits.

✓ Syphilis is a chronic disease which is fatal only after the lapse of a considerable time, usually many years, and then, as it were only by action on the blood or tissue.

by the blood of places

but constitutional disturbances are very slight. It is probable that

✓ syphilis is never a self-curing disease, but even under natural conditions, and without treatment, the syphilitic may have periods of intermission when no signs or symptoms of the disease are apparent. ✓ In a longer or shorter time, however, the latent spirochaetes will again become active and produce definitely syphilitic lesions.

The problems of immunity in syphilis are full of difficulties. The disease, as has been said above, is not self-healing, that is, ✓ the presence of spirochaetes in the human body does not lead to the development of antibodies which are capable of destroying them. Nevertheless it is found that a man suffering from syphilis cannot again be infected with the disease, or at least does not develop a primary lesion, ✓ but the syphilitic who has been cured may be again infected; indeed, a definite re-infection is regarded as one of the strongest proofs of complete cure.

The Wassermann and ✓ do not become positive until some time after the appearance of the chancre: in the absence of the chancre, and are positive in practically every case of secondary syphilis and in the majority of tertiary cases. In a high proportion of late ✓ syphilitic conditions of the central nervous system, they are positive in the blood or in the cerebro-spinal fluid, or in both. They are positive in the case of the majority of children presenting symptoms of congenital syphilis. As has been said before, remissions occur in the disease, and during these periods, when no

evidence of active syphilis may be detectable, the reactions may

observed before a true cure is claimed. ✓ A patient in the primary or secondary stages who has received a full course of treatment and whose blood has given a negative reaction at intervals of 3 months over a period of 2 years is almost certainly cured. In old standing cases, which have had no treatment or insufficient treatment, it may be impossible to reduce the reactions to negative. We cannot be dogmatic as regards these persistently positive reactions, but it may be that such results do not, of necessity, mean a continued presence of the spirochætes in the body.

✓ For the diagnosis of the disease, the best method is the discovery of the organism. If a chancre is present, it should be well washed with saline to free it from contaminating organisms, and should then be vigorously rubbed with a piece of gauze. This operation is rather painful, but less so than the scarification usually recommended. ✓ The effect is to promote a flow of serum which may at first be bloody but later becomes clear. In this serum the spirochætes are usually present in considerable numbers. They are best looked for with the dark field condenser and are recognised by their extreme thinness, their sharp, deep, regular spirals and their

characteristic movements. In the case of genital sores, other spirochaetes may be seen, but these present little difficulty, as they are, for the most part, thicker with coarser and less regular curves than *Treponema pallidum*. In lesions about the mouth, however, certain of the saprophytic mouth spirochaetes, which very closely resemble *Treponema pallidum*, may cause great difficulty in making a diagnosis.

In the absence of a dark field condenser, the organisms may be stained, preferably by Fontana's method, or preparations may be made by the Indian ink or nigrosin methods. Examination in the living condition is, however, always better. Where tissue can be obtained for sections, Levaditi's or Warthin's method of staining, which are too complicated to be described here, give the best results.

In the secondary stage, where material can be obtained from a skin lesion, the organism may be searched for, but more usually the diagnosis is confirmed by one of the serological tests. Where a full positive result is obtained on more than one occasion, and where yaws can be excluded, syphilis is almost certainly present.

appearance of lesions and there is good evidence that it is capable, in some cases, of effecting a complete cure of the disease. The majority of cautious venereologists, however, still use penicillin in conjunction with the well tried drugs which have, as their active constituents, either arsenic or bismuth.

Yaws or Framboesia is a non-venereal disease occurring in certain tropical countries. It is due to infection with Trep pertenuis, an organism almost identical with *Trep. pallidum*. Despite many points of similarity, including the occurrence of a positive Wassermann reaction, the two diseases must be regarded as distinct since they may co-exist in the same person.

The dermal manifestations of the early

THE LEPTOSPIRÆ

Leptospira icterohæmorrhagiæ

Inado and Ido, 1914

Leptospira icterohæmorrhagiæ, the causative organism of Weil's disease, measures 6μ to 12μ and is about 0.15μ in thickness. It is so fine and its spirals are so small, regular and close-set (0.5μ by 0.5μ) that, unless examined very carefully with high magnification, it may appear to consist of a chain of granules. It also exhibits a coarse waviness. Its greatest point of distinction from other spirochætes is that one or both ends are sharply curved, forming terminal hooks. In wet preparations it is seen to whirl and spin at great speed.

Lepto. icterohæmorrhagiæ can be cultivated in dilute rabbit serum (1 part serum to 7 parts glass distilled water). Since it is microaerophilic rather than anaerobic, the surface of the medium should not be covered with oil. The optimum temperature of growth is from 25° to 30° .

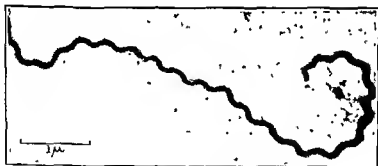


FIG 75.—*Leptospira icterohæmorrhagiæ*.
(Electron microscope)

Weil's disease, or epidemic jaundice, has been observed in many parts of the world. It is characterised by the occurrence into spite cases.

characteristic movements. In the case of genital sores, other spirochaetes may be seen, but these present little difficulty, as they are, for the most part, thicker with coarser and less regular curves than *Treponema pallidum*. In lesions about the mouth, however, certain of the saprophytic mouth spirochaetes, which very closely resemble *Treponema pallidum*, may cause great difficulty in making a diagnosis.

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In the secondary stage, where material can be obtained from a skin lesion, the organism may be searched for, but more usually the diagnosis is confirmed by one of the serological tests. Where a full positive result is obtained on more than one occasion, and where yaws can be excluded, syphilis is almost certainly present. A confirmatory test, not strictly bacteriological, is the rapid disappearance of skin lesions when one of the arsenical compounds is administered.

Treatment of syphilis with penicillin rapidly leads to a disappearance of lesions and there is good evidence that it is capable, in some cases, of effecting a complete cure of the disease. The majority of cautious venereologists, however, still use penicillin in conjunction with the well tried drugs which have, as their active constituents, either arsenic or bismuth.

Yaws or Framboesia is a non-venereal disease occurring in certain tropical countries. It is due to infection with Trep. pertenue, an organism almost identical with *Trep. pallidum*. Despite many points of similarity, including the occurrence of a positive Wassermann reaction, the two diseases must be regarded as distinct since they may co-exist in the same person.

peutically.

The chief source of infection of man is the rat, which acts as a reservoir of the leptospiræ, the organisms lodging in the kidneys and being excreted in the urine. A high proportion of wild rats in all countries harbour the parasite. Human infection may occur through eating food contaminated with rat urine, but an indirect method of spread is more common. The leptospiræ can survive in water for a considerable time; it is even possible that, under suitable conditions, they may grow in it. Human beings may

miners, and those employed in cleaning fish and in preparing tripe. Cuts and abrasions of the skin probably facilitate the penetration of leptospiræ into the tissues. *Lepto. icterohæmorrhagiae* is not the only *Leptospira* found in water. *Lepto. biflexa*, which differs from *Lepto. icterohæmorrhagiae* both antigenically and in being devoid of pathogenicity for man and animals, is commonly present in the slime of fresh water ponds, lakes and rivers and is occasionally found about water taps.

Another *Leptospira*, *Lepto. canicola*, causes uræmia in dogs or may be excreted in the urine of healthy dogs which are carriers. In man, the disease caused by this organism is similar to Weil's disease but is usually milder and jaundice rarely occurs.

Diagnosis of the disease is usually made by the intraperitoneal inoculation of a guinea-pig with the patient's blood in the first few days, or, later, with the centrifuged deposit of the patient's urine. Since some human strains have little pathogenicity for

The mortality varies greatly with age and in different countries, the average being about 12 per cent.

The parasites are present in the blood stream during the first few days of the disease, but as the number is small, direct examination may fail to reveal their presence, which is most easily detected by animal inoculation or by direct culture. When the organisms can no longer be found in the blood, they may be present, often in considerable numbers, in the internal organs, particularly in the liver, kidneys and adrenals. Later, they dis-



FIG 76 — *Leptospira icterohamorrhagiae* IN SECTION OF KIDNEY OF AN INFECTED GUINEA-PIG ($\times 1200$).

appear from the organs, but may continue to be excreted in the urine for several weeks after convalescence.

The guinea-pig may be infected with most strains as a result of either intraperitoneal inoculation or scarification of the skin. The animal develops jaundice and death occurs in about 10 days after inoculation. Hæmorrhages are found subcutaneously and in the lungs and intestine, and the spleen is enlarged. The organisms are numerous in the kidneys, adrenals, and liver.

The nature of the immunity developed in man against the

having
bacillus
 are very
 number of loose, irregular spirals. ✓ The practically constant
 presence in large numbers of the two organisms in clinical Vin-
cent's angina is suggestive of their ætiological importance; but
 exactly similar bacilli and spirochætes may be found in many
 normal mouths, or in the neighbourhood of teeth which are



FIG 77—SPIROCHÆTES AND FUSIFORM
 BACILLI FROM THROAT OF A CASE OF
 VINCENT'S ANGINA (× 950)

carious or around which pyorrhœa alveolaris is present, but here
 they are rarely so numerous as in Vincent's angina.

Both the bacilli and the spirochætes have been cultivated under
anaerobic conditions, and although it has been claimed that the
 ✓ bacilli develop into the spirochætes, this has not been substanti-
 ated. They are almost certainly distinct organisms which thrive
symbiotically, and which of the two, if either, is to be regarded
as causative, or whether the combination is responsible for the
condition is as yet undecided. A similar combination of the two

animals, direct culture from the same materials should be attempted. ✓ Microscopic examination of the deposit from the urine with the dark field condenser is often sufficient, but care must be exercised since other spirochætes may be present. The characteristic hooked ends of the leptospiræ are, however, of great diagnostic importance. Serological methods of diagnosis are also available. The serum of a patient agglutinates *Leptospira* after 2 hours at 37° or in formal-

the twentieth day, the titre of the serum may be 1 : 100,000. Titres of less than 1 : 400 are of doubtful significance. An alternative method is the adhesion test in which, after the fresh unheated serum of the patient has been kept in contact with cultures of the organism and of *Bact. coli* at 37° for 30 minutes, it is found that the bacilli are firmly adherent to the leptospiræ.

Certain other species of *Leptospira* are pathogenic for human beings. Seven-day fever of Japan is caused by *Lepto. hebdomadis* and swamp or mud fever of eastern Europe by *Lepto. grippotyphosa*. These leptospiræ are antigenically distinct from *Lepto. icterohæmorrhagica* and *Lepto. biflexa*.

OTHER SPIROCHÆTES

In Vincent's angina, a condition of the throat, pharynx or mouth, in which a false membrane with ulceration occurs, as also in acute ulcerative gingivitis, a very characteristic microscopic picture is obtained by staining films made from the lesion with gentian violet or carbol-fuchsin. ✓ Large numbers of fusiform bacilli, the *Fusobacterium plauti-vincenti* (*Fusiformis fusiformis*), are seen associated with many spirochætes, *Borrelia vincentii* (*Treponema vincenti*). ✓ The bacilli, which measure from 3μ to 10μ in length by about 0.7μ in the centre, taper sharply towards either end. They are usually straight or slightly curved. They stain with some difficulty and carbol fuchsin gives the best results. Most characteristically the stain is not taken uniformly, the bacilli presenting a striped or beaded appearance, a very common form.

Rat-bite fever, caused by *Spirillum minus*, must be distinguished from a completely different disease, spread in the same way, the causative organism of which is *Actinomyces muris* (*Streptobacillus moniliformis*), a normal parasite about the mouth in rats.

Penicillin is effective in the treatment of both varieties of rat-bite fever.

growth occurs along the track of the wire, and from this very delicate offshoots pass into the medium. On moist agar, the growth is in the form of a thin film which spreads rapidly over the surface. Hæmolysis is produced in blood-agar. Gelatin is liquefied and there is some digestion of egg white. Indole is produced. No sugar is fermented.

Spores form in from 24 to 48 hours at body temperature, but much more slowly at air temperature. In the wet state, the spores withstand a temperature of 80° for several hours, but are usually destroyed in less than a quarter of an hour by boiling. Like other



FIG 79 — *Cl tetani* FROM AGAR CULTURE ($\times 800$)

spores, they are much more resistant against heat in the dry state. Kept dry, in the dark and in the cold, they have been found to be still viable after 18 years. They resist antiseptics well, and may survive 10 hours' exposure to 5 per cent phenol; 1 per cent. silver nitrate solution, however, kills them in one minute, and chlorine is also active against them.

CHAPTER XLII

TOXIN-PRODUCING CLOSTRIDIA

Bergey's Manual lists 61 species of the genus *Clostridium* which consists of anaerobic, spore producing bacteria. We are concerned only with the small number of these which play a part in the causation of human disease. The two species considered in this chapter are separated from the others because they cause disease

from the local lesion to the central nervous system. *Cl. botulinum* produces its toxin not in the body at all, but in food which is subsequently eaten.

Clostridium tetani Nicolaïer, 1884

The tetanus bacillus measures from 2μ to 5μ in length by 0.3μ to 0.7μ in thickness. It is a straight, round-ended bacillus which is most commonly found singly or in pairs, but long chains of bacilli occur in cultures. It is sluggishly motile and has no capsules. Spores are produced both in the bodies of animals and in culture. They are terminal and spherical or oval, measuring from 1μ to 1.5μ in diameter, and the bacillus with its spore resembles a drum-stick in appearance. The bacillus stains readily and is Gram positive.

Cl. tetani is an obligatory anaerobe. Its optimum temperature of cultivation is that of the body, but growth goes on slowly at 20° . In broth, a fine turbidity, with later a deposit at the bottom of the tube, is produced. Meat broth is unaltered, no digestion of the meat and no discoloration being detectable. In an agar stab,

growth occurs along the track of the wire, and from this very delicate offshoots pass into the medium. On moist agar, the growth is in the form of a thin film which spreads rapidly over the surface. Hæmolysis is produced in blood-agar. Gelatin is liquefied and there is some digestion of egg white. Indole is produced No sugar is fermented.

Spores form in from 24 to 48 hours at body temperature, but much more slowly at air temperature. In the wet state, the spores withstand a temperature of 80° for several hours, but are usually destroyed in less than a quarter of an hour by boiling. Like other



FIG. 79—*Cl. tetani* FROM AGAR CULTURE ($\times 800$).

spores, they are much more resistant against heat in the dry state. Kept dry, in the dark and in the cold, they have been found to be still viable after 18 years. They resist antiseptics well, and may survive 10 hours' exposure to 5 per cent phenol, 1 per cent silver nitrate solution, however, kills them in one minute, and

some countries, in the faces of man. The intestine is probably its normal habitat, where it lives as a harmless saprophyte.

The disease which it produces, tetanus, occurs most frequently in man and in the horse, but the majority of mammals are susceptible to artificial inoculation. In man the disease is usually the outcome of a wound, particularly of a deep stab or penetrating wound or one which is much lacerated and contused. The presence of dirt, of foreign bodies or of other bacteria, is almost to be considered as requisite for the production of tetanus. A number of cases of tetanus have been traced to the use of improperly prepared catgut in operations. The type of the disease which was formerly known as "idiopathic" was almost certainly due to a slight wound, not then regarded as having any connection with the disease. The period of incubation may be as short as a few days or may be months. The bacilli or their spores may lie dormant in the tissue until some later injury, such as operative interference, creates an environment suitable for their development. The organism has been isolated from the site of an old wound more than 2 years after its infliction. Another form of the disease, that occurring in the umbilical cord, may be present in the presence of other bacteria. The bacilli and practically no infection on a few occasions, been known which they produce on the body, the terrible tonic spasms of muscles, are due entirely to the exotoxin produced by them. This toxin is taken up by the end organs of the motor nerves and passes up their axis cylinders to the central nervous system. Some passes also to the blood stream, but can reach the central nervous system only by way of the peripheral nerves. The spasms are central in origin, and are due to the action of the toxin on the nerve cells in the cord. The affinity possessed by nerve cells for toxin is shown by the fact that, by mixing toxic broth with brain substance, the former is completely deprived of its poisonous properties. The disease is very fatal unless modified by the

previous or almost coincident administration of antitoxic serum.
 → The shorter the period of incubation, the worse is the prognosis.
 The post-mortem changes are very slight, some congestion of the
 internal organs and of the central nervous system, with a degenera-

local spasms in the neighbourhood of the site of injection. These
 soon become general and death occurs within a few days. It is
 found that no matter how large a dose of toxin is given, an incuba-
tion period, at least 8 hours in the mouse, elapses before symp-
 toms appear. It is probable that this represents, in part at least, the
time necessary for the toxin to travel along the nerves. The
 inoculation of one of the smaller animals either with cultures of
 the bacillus or with soil or other material containing bacilli
 invariably causes typical tetanus to develop in the animal. If,
 however, the spores are carefully freed from toxin by washing and
 injected suspended in saline, or if the toxin present in the culture
 is destroyed by heating to 80° , the animal frequently escapes. The
addition of other bacteria, foreign bodies such as a splinter of
wood or earth, lactic acid, calcium chloride, guinine, or of their
own toxin is sufficient to cause the disease to appear. The explana-
 tion is that if spores are injected alone they lie dormant, as too
much oxygen is present to permit them to vegetate. The tis-
 sue debilitants mentioned cause either local necrosis, or, at
 least, interfere with normal blood supply, so that the oxygen
tension of the tissues is lowered to a level which allows of develop-
ment of the bacilli. Where spores are injected in one locality in
 an animal's body and a tissue debilitant in another, the bacilli
 grow only at the site of the debilitant. Spores deposited there by phago-
cytic cells that a badly
 with dirt, is a more likely precursor of tetanus than is a clean
 wound inflicted with a sharp instrument.
 A suitable broth and anaerobic conditions are necessary for
 the production of toxin in vitro. The maximum yield of toxin is

found at the end of from 7 to 14 days' incubation, although little growth occurs after 48 hours. ✓ The toxic broth is freed from bacilli by filtration through a Berkefeld or Chamberland filter, and, after the addition of 0.5 per cent. phenol, may be preserved in the dark at a low temperature and protected from oxygen. The reverse of these conditions (the presence of oxygen, high temperature and bright light) causes a rapid fall in the toxicity of the broth. A temperature of 65° completely destroys the toxin in less than 1 hour. ✓ The toxin may be concentrated by precipitation, the broth being saturated with ammonium sulphate and the precipitate freed from the salt by dialysis. The dry solid obtained by evaporation *in vacuo* is not pure toxin, but it is very rich in toxin, and its strength is retained for long periods if kept dry and in the cold. Tetanus toxin is a most powerful poison, as small an amount as 0.00001 ml. of the filtrate being sufficient to kill a mouse. It

igram of dry toxin

ministered subcutaneously, intramuscularly or intravenously. It is, however, without effect when given by the mouth, some being destroyed by gastric and intestinal secretions and by the action of intestinal bacteria and some being excreted unchanged with the faeces.

Antitoxic serum is prepared by the immunisation of horses in a manner similar to that used for the preparation of diphtheria antitoxin. The potency of a new batch of antitoxic serum is determined by comparing its capacity to neutralise tetanus toxin with that of a standard serum. ✓ The amount of antitoxin present in a serum is expressed in units per ml. and the dose administered to a patient is stated in units, irrespective of the volume of serum.

The great success of the serum treatment of diphtheria led to corresponding hopes in connection with tetanus antitoxin, but these expectations were not realised when the serum was tested. ✓ For this there are many explanations: the disease presents no striking local lesion which allows of its diagnosis before symptoms of intoxication are present, the toxin has a greater affinity for nerve cells than for its antitoxin; the nerve cells are more suscept-

ible to damage than the sturdier muscle or gland cells attacked by diphtheria toxin; diphtheria toxin circulates in the blood where it can be acted upon by injected antitoxin, while tetanus toxin is removed from the circulation by the nerve end organs, whence it passes by way of the nerves to the nerve cells, and in this route it is but slightly exposed to antitoxic action. Animal experiments have shown that, if the amount of antitoxin be found which is sufficient to protect against a given dose of toxin when the two are administered together, the antitoxin must be enormously increased when even a few minutes supervene between the administration of the toxin and the antitoxin. The moral is that antitoxin, to be really successful, should be given before symptoms appear, that is, that a prophylactic injection should be made in the case of every dirty wound, just as to-day anti-diphtheria serum is given in every suspicious throat case. The prophylactic dose should be 3000 units.

In the case of severe wounds which are slow in healing, serum should be injected twice or thrice at weekly intervals. Without serum the mortality from tetanus is 80 to 85 per cent, and although even with the free use of antitoxin declared tetanus is a very fatal disease, nevertheless serum should be given. On account of its slow rate of absorption, the subcutaneous or intramuscular routes are unsuitable, except to act as reservoirs to maintain the concentration of antitoxin in the body. Every moment is of importance and large amounts of serum should be given both intrathecally and intravenously. In all two hundred thousand units or even more may be administered and, although death frequently occurs despite this, the successes have been sufficiently numerous and, in a number of cases, striking to warrant the fullest use of antitoxin, even in apparently hopeless cases.

Soldiers and others whose occupation renders them specially liable to contract tetanus, may be actively immunised by the injection of tetanus toxoid (toxin rendered non-toxic by the action of formalin) on two occasions 6 weeks apart. To maintain the amount of antitoxin in the blood at a sufficiently high level (about 0.02 unit/ml) to protect against the disease, a boosting dose of toxoid should be injected every year. During the war

of 1939-45, complete reliance was placed on this active immunisation by the authorities of the American and Canadian armies and antitoxin was not normally administered to the wounded. The incidence of tetanus was very low. In the United Kingdom forces during the same war, active immunity was reinforced by the injection of antitoxin in the case of all severely wounded men. The success of the prophylaxis of tetanus is shown by the fact that, during the 6 years of war, only 23 cases of tetanus occurred among the 239,052 wounded United Kingdom soldiers, an incidence of 0.1 per 1,000. A considerable proportion of the 23 had either completely escaped inoculation with tetanus toxoid or had not been adequately inoculated. The incidence of 0.1 per 1,000 compares favourably with that of 1.47 per 1,000 wounded, which was the experience of the British armies on the Western Front in the war of 1914-18.

Diagnosis of the declared disease is usually a clinical matter, but the finding of typical drum-stick bacilli in a wound is suggestive, although there are many other bacilli morphologically similar to *Cl. tetani*. The organism should be isolated in pure culture which will be facilitated if the material be heated to 80° for 10 minutes to destroy non-spore-forming bacteria and inoculated into the condensation water of an agar slope. After 24 hours incubation anaerobically a pure culture may frequently be obtained by picking from the filamentous edge of growth which may have spread a considerable distance up the agar surface. The identification is completed if the isolated organism causes tetanus in animals inoculated with it. Inoculation of animals with impure cultures is not always successful, as the other bacteria present may destroy tetanus toxin.
✓ Since there are ten antigenic types of tetanus bacilli (all of which produce the same toxin) agglutination tests are of little value for the identification of the organism.

Clostridium botulinum (*Botulus* = a snake)
 Van Ermengem, 1896

Cl. botulinum is a large, straight, rounded-ended, slightly motile bacillus, measuring from 4 μ to 6 μ by 0.9 μ to 1.2 μ , which is a subterminal organism with motile flagella.

found singly or, more rarely, in short chains. The bacillus stains readily and is Gram positive. It produces oval spores which are only slightly thicker than the bacilli and are situated at or near the ends.

The organism is strictly anaerobic, and grows fairly readily on ordinary media of neutral or slightly alkaline reaction, the presence of glucose greatly assists growth. The optimum temperature of cultivation is about 25°. On solid medium, the colonies are yellow, translucent and coarsely granular, and later become more opaque and brownish. On blood-agar, it is haemolytic. In stab-cultures in gelatin or agar containing glucose, the medium is cracked and split by the evolution of gas. Gelatin is rapidly liquefied. Glucose and maltose are fermented. In meat medium there is some digestion and darkening of the meat. All cultures have a rather unpleasant rancid odour, due to the production of butyric acid.

The resistance of the spores to heating is variable. The majority of bacteriologists have found them less resistant than those of other sporing bacteria, being usually killed in 1 hour at 85° and rapidly at 100°. Some highly resistant spores have, however, been described which survived boiling for several hours and 120° for 20 minutes. This inconsistency may, in part at least, be due to the fact that acidity of the medium has a marked effect in lowering the lethal temperature, as has also the presence of salt.

The natural habitat of the organism is not known with certainty, but it is probable that it carries on a saprophytic existence on vegetable matter in the soil. Its importance to man is in its occurrence in various articles of diet in which it forms an active poison. The nature of the food is very varied, but practically all forms have one thing in common—they are preserved. The disease produced (botulism) derives its name from some outbreaks due to eating sausages, but ham (usually eaten raw), tinned meats, tinned fruit (apricots and pears), vegetables (asparagus, peas, beans and olives), fish and other foods have been incriminated. *corn, spinach*.

The bacillus itself does not appear to be pathogenic, and multi-

plies little, if at all, in the alimentary canal. It can only rarely be isolated from the faeces of victims, and only in one or two cases has it been found in the excreta.

for the production of this toxin: a warm air temperature, absence of oxygen and suitable food material (not necessarily animal) are essentials, and these are frequently to be found in large sausages, smoked hams and canned foods. The growth of the bacilli usually produces little or no change in the food, which is never apparently "bad", although a slightly rancid odour may be detectable.

Botulism differs from the other forms of food-poisoning in that gastro-intestinal symptoms are very slight. The action of the toxin is chiefly on the cranial nerve centres, and this is responsible for the most prominent symptoms, involvement of the eye muscles with protrusion of the eyeballs, ptosis, loss of accommodation, dilated pupils, as well as aphonia and dysphagia. Disturbances of the flow of saliva, either dryness of the mouth or salivation, are commonly present. Constipation and retention of urine are sometimes noted; fever is generally absent and consciousness is retained. Symptoms usually appear within 24 hours of eating the food and death occurs 4 to 8 days later. Death is due to involvement of the respiratory or, more rarely, of the cardiac nerve centres. The severity of the symptoms depends on the amount of food (and therefore toxin) consumed. This fact may explain the differences in the fatality rates in different outbreaks which average about 65 per cent.

✓ The toxin of *Cl. botulinum* is the most poisonous substance known. A filtrate of a broth culture may kill a guinea-pig in a dose of 0.000001 ml. and it has been calculated that less than one-hundredth of a milligram of the dry toxin would kill a man. The toxin is fairly resistant to heat and may withstand exposure to 80° for some time. It is, however, rapidly destroyed at 100°. The toxin of *Cl. botulinum* differs from those of *Cl. tetani* and

2. action of toxin uncertain but the Central Nervous
involved peripherally rather than centrally. 433
is large a dose, however, being necessary to kill the animal when
given in food as when injected subcutaneously.

It should be appreciated that botulism is a poisoning and not
an infection. Botulism is classed as a bacterial food poisoning
merely because the poison is of bacterial origin. It is completely
different from bacterial food poisoning due to one of the salmonel-
lae in which the organism usually multiplies and produces its
toxin within the body.

By the immunisation of animals it is possible to prepare an
antitoxin which completely neutralises the toxin. The antitoxin
has been used therapeutically in the disease in man in a few cases,
but it is of doubtful value. It is conceivable, however, when a
number of persons have partaken of the same food, and one
or more have developed symptoms of botulism, that the adminis-
tration of the serum to those unaffected might have some pro-
tective action.

At least five types (A to E) of *Cl. botulinum* have been distin-
guished. These display minor differences in morphology and
cultural characteristics and are antigenically distinct. A much

in man has occurred as a result of eating food in which *Cl.*
botulinum of Types A, B or E have grown. Types C and D pro-
duce a variety of diseases in lower animals.

Diagnosis is usually made on clinical grounds and, from the
character of the disease, presents little difficulty. Bacteriological
confirmation is not generally possible by the isolation of the
organism from the body, but cultures should be made from all
suspected food. Glucose broth and glucose gelatin are the media
of choice, and, by heating to 60° for half an hour, the food may
be freed from the majority of non-sporing bacteria

Prophylaxis is chiefly a matter of the hygienic preparation of
foodstuffs. Since the toxin which is the cause of the disease is
thermolabile, the heating of any food, which may be suspected,

Dormancy and heat-resistance are features. HB—FF

to 100° for a few minutes immediately before use, will make it quite safe. ✓ Anaerobic conditions are of course most perfect in canned and bottled foods, and these should be rejected if they are not in perfect condition, as judged both by the eye and by the nose. While a large number of cases have been due to canned or bottled foods, it is right to point out that these have usually been home-prepared. ✓ Factory methods of sterilisation are more accurately carried out than are those of the private house. Since botulism is a very rare condition, the possibility of its occurrence should not rule out of the dietary useful foods, of which many millions partake in safety for every one who suffers from the disease. ✓ If, however, there is the slightest suspicion of the food, it should not be eaten. Death has resulted from eating half an olive. In the case of canned foods, the cans may be blown as a result of contamination of food with organisms in situ.

CHAPTER XLIII

ANAEROBIC BACTERIA OF WOUNDS

The five species of the genus *Clostridium* to be considered in this chapter are almost completely devoid of infectivity. They do not spread from person to person as does *Salm. typhi* or *Strep. pyogenes*. They can multiply in the tissues of the body only when they are implanted in an area which has suffered considerable mechanical damage and are there assisted to establish themselves by the presence of extraneous material. These organisms are, therefore, of importance in human pathology only because of the serious effects they produce in wounds and particularly in the extensive wounds inflicted in warfare.

Identification of the pathogenic clostridia is generally more difficult than is the case with the majority of the aerobic bacteria with which we have dealt. Apart from the inherent difficulties of anaerobic culture, other difficulties arise from the occurrence of atypical, pleomorphic forms, the tendency of clostridia to symbiosis, the similarity of the colonial forms of different species, the variability in the fermentative capacity of the same species and, usually, the antigenic complexity of the species.

The clostridia with which we are concerned have, as their main habitat, the soil and the intestines of man and animals. They can probably multiply indefinitely in decaying vegetable matter in the soil without the necessity of assuming a parasitic existence in the intestines but in heavily manured soil, in which they are usually present in very large numbers, their main source is excreta. Their ability to produce spores enables them to survive absence of food and water for long periods.

The species of *Clostridium* associated with wounds may, as a matter of convenience, be divided on biochemical grounds into two groups—the saccharolytic and the proteolytic. The species in the first group, *Cl. perfringens*, *Cl. septicum* and *Cl. novyi*, are

active fermenters of carbohydrates with little power of digesting proteins. In the second group, *Cl. histolyticum* and *Cl. sporogenes* are capable of breaking down proteins but have only slight

Clostridium perfringens (*Cl. welchii*)

Welch and Nuttall, 1892

This organism is a large, Gram positive bacillus with square-cut or slightly rounded ends. Most characteristically it measures



FIG 80--*Cl. perfringens* FROM AGAR CULTURE ($\times 800$).

from 4μ to 8μ by 0.8μ to 1.2μ , but occasionally short, almost coccoid, forms as well as filaments, are seen. It is usually found singly, or in pairs; chains are rare except in old cultures. It is motile and possesses no flagella. Capsules are present in

only when

oval spores

are situated

the tissues

they are

not produced in media containing any carbohydrate fermented by the bacillus.

Cl. perfringens is a strict anaerobe. Growth is not abundant except in the presence of glucose or other utilisable carbohydrate. On blood-agar it produces round, smooth, even colonies surrounded by zones of hæmolysis. In meat broth there is some clouding, the meat becomes slightly pinkish in colour, and gas is evolved abundantly. There is no evidence of digestion and the odour is sour, owing to the production of butyric acid, but not putrid. A very characteristic change is produced in whole-milk cultures. There is

Indole is not produced. The organism is very feebly, if at all, proteolytic, but is actively saccharolytic, glucose, maltose, lactose, saccharose and other carbohydrates being fermented with vigorous gas evolution.

The Nagler reaction, which is strongly positive in the case of *Cl. perfringens*, particularly of Type A, is of considerable value in the identification of the organism. A few other clostridia give a weakly positive reaction.

The reaction may be elicited by inoculating "inactivated human serum" or a clear solution of egg yolk with the organism and incubating over night. The development of opalescence with the appearance of a fine, yellow curd, which tends to rise to the surface and is most marked in the egg yolk preparation, is a positive result. The reaction is due to lecithinase produced by the organism acting on lecithin in the serum or egg yolk. For the reaction to occur, calcium and magnesium ions

Lecithinase activity of the α toxin splits the Lipo-protein of Serum.



FIG 81—STORMY FERMENTATION OF MILK BY *Cl. perfringens* ($\times \frac{1}{2}$)

must be present and, for this reason, serum must be used and not citrated or oxalated plasma. ✓ The reaction may also be demonstrated by culturing the organism on a plate of agar containing 20 per cent. of human serum. ✓ Zones of opalescence surrounding the colonies indicate a positive result. ✓ Antitoxin added to the tube or spread over half the plate

of resistance and the organism dies fairly rapidly in cultures in which glucose or other fermentable sugar is present, both owing

FIG 82—*Cl perfringens* IN MUSCLE
(× 950)

to the absence of spores and to the acid produced. ✓ The spores are resistant and may withstand boiling for a few minutes.

The bacillus is widely distributed in nature, being found in the soil, in water, in milk and in the faeces of many species of animals,

being the cause of the toxæmia which occurs as a result of acute

intestinal obstruction, peritonitis and, occasionally, appendicitis. *Cl. perfringens* has higher invasive powers than most of the other clostridia and may, not infrequently, be isolated from the blood during life. The most important demonstration of the pathogenic power of *Cl. perfringens* will be dealt with later when gas gangrene is considered.

Four types, A, B, C and D of *Cl. perfringens* have been differentiated. Of these only one, Type A, is associated with disease in man. The others are responsible for a variety of diseases in lower animals. Seven distinct toxins are produced by the four types of *Cl. perfringens*, no more than five of these have been produced by

and acts as lecithinase. As compared with the toxins of *Cl. tetani* and *Cl. botulinum*, the toxin produced by *Cl. perfringens* of Type A is rather feeble, its M.L.D. for the mouse being about 0.25 ml. Many strains of Type A also produce hyaluronidase.

Clostridium septicum (*Vibrio septique*)

Pasteur and Joubert, 1877

Cl. septicum is a straight or slightly curved bacillus, measuring from 3μ to 8μ by 0.4μ to 0.8μ , but very much longer forms are frequently seen, both in the tissues and in culture. It is found lying singly, in pairs or in chains, and occasionally in long filaments in which it is doubtful whether subdivisions into bacilli are present. It is feebly motile, but under aerobic conditions is non-motile. No capsules are produced. The organism stains easily and young forms are Gram positive, but in older cultures it may be Gram negative. Oval spores, slightly thicker than the bacilli, are produced and are situated either centrally or subterminally. Spores are rarely produced in the living body but are found in cultures, especially in a medium containing body fluids and with a minimum of utilisable carbohydrate. Two kinds of involution forms are observed; the first of these consists of long filaments, which are most regularly found on the surface of

must be present and, for this reason, serum must be used and not citrated or oxalated plasma. The reaction may also be demonstrated by culturing the organism on a plate of agar containing 20 per cent. of human serum. Zones of opalescence ...

colonies indicate :

or spread over ha

lecithinase and pri

The vegetative

resistance and the organism dies fairly rapidly in cultures in which glucose or other fermentable sugar is present, both owing



FIG. 82 — *Cl. perfringens* IN MUSCLE
($\times 950$).

to the absence of spores and to the acid produced. The spores are resistant and may withstand boiling for a few minutes.

The bacillus is widely distributed in nature, being found in the soil, in water, in milk and in the feces of many species of animals,

Clostridium novyi (*Cl. oedematiens*)

Novy, 1894, Weinberg and Sequin, 1918

This bacillus, which measures from 3μ to 10μ by 0.8μ to 1.0μ , resembles in appearance *Cl. perfringens*, but is usually rather longer. It occurs singly or in pairs, but long chains may be present in culture. Curved (C) forms are frequently seen, and a few of such form
it is

the bacilli and subterminal, are freely produced in culture, even in the presence of fermentable carbohydrate. In young cultures, the bacilli are Gram positive, but later fail to retain the stain.

It is one of the strictest of the anaerobes and care is required for its cultivation. In glucose broth a uniform turbidity is first produced, but in a short time the bacilli fall to the bottom of the tube, and later the culture becomes clear owing to autolysis. Glucose and maltose are fermented, with the production of acid and gas, but not lactose or saccharose. Meat broth is not darkened, but the meat may acquire a slightly pinkish colour and a few bubbles of gas may be evolved. Gelatin is slowly liquefied. Indole is not produced. The organism is hæmolytic. The spores may remain viable after 30 minutes' exposure to 100° in the wet state.

A characteristic feature of *Cl. novyi* is the production by it of a gelatinous oedema as a result of intramuscular inoculation of experimental animals. The toxin produced by this organism, which is considerably more powerful than those of *Cl. perfringens* or *Cl. septicum*, may have a M.L.D. for the mouse of 0.0002 ml.

Clostridium histolyticum

Weinberg and Sequin, 1918

Cl. histolyticum measures from 2μ to 6μ by 0.5μ to 0.8μ . It is most commonly found as a straight or slightly curved, non-capsulated bacillus, singly, in pairs or, much less frequently, in short chains. It is actively motile. Spores are readily produced in cultures and are oval, wider than the bacilli and sub-terminal

the liver and other abdominal organs when the peritoneal cavity has been invaded in animals experimentally inoculated; [✓]in the other, known as the navicular or citron form, the bacilli are short, thick in the centre and tapering towards the ends, or almost oval with bulged ends, the outline resembling closely that of the bed bug. In this form it is common to observe darker staining dots at one or both ends. The bacillus is a moderately strict anaerobe. [✓]Growth in meat broth results in the meat turning pinkish in colour, without any blackening or signs of digestion. The odour is sour owing to the production of butyric acid, and some gas may

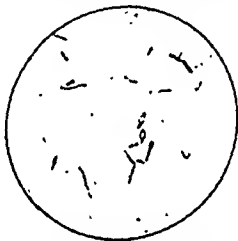


FIG. 83 — *Cl. septicum* FROM BROTH CULTURE (X 850).

be formed. In milk, a clot is produced and gas evolved, but the alterations are never so striking as in the "stormy fermentation" of *Cl. perfringens*. Gelatin is liquefied. Glucose, lactose, and maltose are fermented, but not saccharose. The organism is less actively saccharolytic than *Cl. perfringens* and is not proteolytic. Indole is not produced. The bacillus is actively hæmolytic. The spores are highly resistant and can survive boiling for some time.

Cl. septicum has considerable invasive powers and has frequently been isolated from the blood stream. Its toxin is moderately powerful, its M.L.D. for the mouse being about 0.005 ml.

restricted, glucose and maltose being the chief carbohydrates fermented.

Cl. sporogenes is widely distributed in nature. It is one of the

Its powers of resistance are so high as to render it difficult to free



FIG. 84—*Cl. sporogenes* FROM BROTH CULTURE ($\times 950$)

media from it, since the spores may resist boiling for 1 hour and even a temperature of 115° in the autoclave for the same time.

Cl. sporogenes does not produce any toxin and its pathogenic powers are limited. Thanks to its powerful proteolytic enzyme, it is able to digest dead tissue but, unlike *Cl. histolyticum*, is without action on living tissue.

Gas Gangrene

The term gas gangrene has been used as the heading of this section, not because it is either accurate or appropriate, but because it is the one still in common use to describe the most serious condition, except tetanus, which can develop in a wound.

Almost every extensive wound, apart from those deliberately

in position. ✓ Young bacilli are Gram positive, older forms failing to retain the stain.

Cl. histolyticum grows fairly readily under anaerobic conditions and is actively proteolytic. Meat is darkened and in the deep frequently liquefied. with the p

the organism may have a slight and slow action. All cultures have a foul and nauseating odour.

✓ The toxin produced by *Cl. histolyticum* is a feeble one, but the organism produces a powerful proteolytic enzyme which is almost unique in its ability to digest living tissue.

Clostridium sporogenes

Metchnikoff, 1908

Cl. sporogenes usually measures from 3μ to 7μ by 0.5μ to 0.8μ , but occasionally long filaments occur. It is a motile organism and does not form capsules. It is found usually singly or in pairs, but some short chains may appear in cultures. It is Gram positive but, like many of the anaerobic bacilli, older organisms

✓ capable of growth in the presence of an amount of oxygen sufficient to inhibit the development of many of the other anaerobic bacteria. In meat broth it grows rapidly and the meat is darkened or even blackened, especially if a piece of iron be present, and subsequently almost completely digested. Some gas is evolved, and the culture has a foul, putrefying odour. In milk there is some coagulation with later digestion. Gelatin, coagulated serum and egg white are all liquefied. Indole is not produced. The organism is actively proteolytic, but its saccharolytic powers are

implanted in the wound develop into bacilli and these multiply, producing their toxins. The characteristic features of gas gangrene are due much more to the local action of the toxins than to any direct action of the organisms

Gas gangrene may be produced by any one of the clostridia mentioned, except *Cl. sporogenes*, and most commonly by *Cl. perfringens*, but commonly two or more species are involved. The toxins of each have slightly different actions and so the pathological picture produced varies somewhat from case to case, depending on the particular species present in each wound.

Two conditions which were formerly grouped together as gas gangrene have been differentiated and named *anaerobic (or clostridial) cellulitis* and *anaerobic (or clostridial) myositis*. Even these terms are not entirely satisfactory, as the *hyperæmia* and *cellular reactions characteristic of inflammation* are absent. The term *myonecrosis*, which has been suggested as an alternative to myositis, is a more apt one. Gas gangrene is inappropriate for

gangrene, there is usually little or no gas.

✓ In anaerobic cellulitis, there is an extensive growth of the clostridia in the depth of the wound and in the tissue spaces; gas, often in considerable amount, is usually present in these spaces, but the muscles appear normal and remain contractile and there is little systemic disturbance. Anaerobic cellulitis is a serious condition but its fatality rate is much lower than that of anaerobic myonecrosis.

✓ of well
py xæmia,
is At first they are dull, pale and œdematous. Later their colour darkens to red or purple and they become non-contractile and friable. The term gas gangrene, if it is to be used at all, should be reserved for this condition which should not be diagnosed unless the muscle fibres are dead. This is the essential criterion and not the presence of gas or of any particular organism. In fact, in myonecrosis, gas

inflicted by a surgeon with full aseptic technique, is contaminated with a variety of bacteria, not infrequently including certain of the clostridia. In the majority of cases, this bacterial contamination, apart from somewhat delaying healing, is not a very serious matter, even when the bacteriological examination reveals the presence of clostridia recognised as pathogenic for man.

Under certain circumstances, however, the clostridia gain the

Clostridia are most likely to flourish and gas gangrene to occur in extensive wounds in which there has been much destruction of muscle, especially of the buttock and thigh, and considerable loss of blood. Other circumstances favouring their development are—

of a compound fracture: the presence in the wound of clothing, soil or other foreign matter: delay in the surgical toilet of the wound: excessively conservative surgery.

The bacteria responsible for gas gangrene are *Cl. perfringens*, and less frequently, *Cl.*

especi-
ne and,

although not primarily responsible, may play a part in enhancing the virulence of those more definitely pathogenic. Again it is emphasised that any one or more of these organisms, with the possible exception of *Cl. histolyticum*, may be found in a wound which is not

The causat
themselves in
able by defective blood supply, by the presence of aerobic bacteria and by other factors which tend to lower the oxygen tension. Suitable nutrients are supplied by extravasated blood and damaged tissues It is probable that salts of calcium and silicon, the time of wounding, favour the

rovided, the clostridial spores

ACTINOMYCOSIS

ends in the tissues. It is an aerobic species. "
A. Maduraz is aerobic Actinomyces bovis - in pus from lunacy
is of non acid fast type Harz, 1877 (Nocardia, Streptothrix)
in Madura foot.

The name Actinomycosis is given to a disease of animals
(chiefly cattle) and man which is characterised by the formation
of granulation tissue accompanied by suppuration. The chief
feature of the process is the occurrence in the lesions of granules
or colonies of the causative organism, the Actinomyces bovis
or ray fungus. These granules, which are frequently yellowish
(sulphur granules) or greyish in colour, may be easily visible
to the naked eye or may be of microscopic dimensions. When
examined unstained with the microscope, it is possible to
make out something of their structure. The central portion is

cor true
brc and
appears to be composed of a rather granular protoplasm sur-
rounded by a delicate sheath. In older colonies the filaments

these fragments were formerly
s almost certainly incorrect.
are filaments which, as they
ie in swollen, homogeneous,
pear-shaped bodies, the so-called clubs. There has been consider-
able controversy as to the nature of these clubs. Some regard

inoculated with Actinobacillus, various acid-fast bacteria and
even inorganic substances. When stained, the filaments are
found to be Gram positive, but the clubs do not retain the stain.
In young colonies the clubs may be exceedingly delicate and be
e human strains of Actinomyces⁴⁴⁷ would be called A.
ile the bovine strain A. bovis. (Erikson, 1940).

is usually either absent or present in very small amount. Myonecrosis, unless promptly and energetically treated, is almost invariably fatal. The most successful treatment may reduce the fatality rate to about 30 per cent.

erobic cellulitis but more
may appear within a few
spreads rapidly along the
muscle bundles involved unless it is checked by almost heroic surgery, local chemotherapy and the general administration of antitoxic sera, penicillin and sulphonamide.

T... available against
 The wound
 cle must be
 excised, every foreign body must be removed, every particle of soil must be washed out. Then, when the whole wound is open, clean and dry, the exposed tissues should be dusted with some bactericidal substance in the form of a fine powder. Strong antiseptics should be avoided. The most valuable of the substances tested have been penicillin, sulphathiazole and proflavine in this order. Since some bacteria are acted upon by one and some by another of these, it is probably best to use a mixture of sulphathiazole and calcium penicillin (5000 units per gram.). Proflavine may be added but not to the extent of more than 1 per cent. as, in higher concentrations, it may cause necrosis—under the circumstances just about the most unfortunate possible result of treatment. Penicillin should be administered in large doses systemically to kill the bacteria, and antitoxin to neutralise their toxins. Powerful antitoxins have been prepared against the toxins of *Cl. perfringens*, *Cl. septicum* and *Cl. novyi*, and these should be administered intravenously. An adequate dose for prophylactic purposes is 9000 units of *perfringens*, 4500 units of *septicum* and 3000 units of *novyi* antitoxins. A mixture of the three antitoxins in these amounts was commonly used with good effect in the later stages of the war of 1939-45.

these media clubs are not produced, but if serum be added there is some swelling of the ends of the filaments ✓ Another satisfactory medium is glucose broth containing a few drops of fresh blood. This may be covered with a thin layer of oil. Colonies like puff-balls form at the bottom of the tube. ✓ The organism grows best at body temperature and but little, if at all, at 20°.

In cattle the parasite affects chiefly the tongue (woody tongue) and jaws, producing lesions in which the predominant feature is granulation tissue rather than suppuration. The nodules are composed chiefly of polymorphonuclear leucocytes, round cells, both

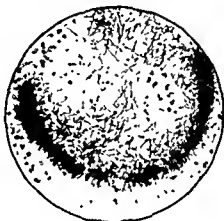


FIG 86—COLONY OF *Actinomyces bovis*
IN LIVER (X 500)

small and large, and plasma cells. There is a considerable proliferation of the surrounding connective tissue, and later the centre of the mass may undergo softening, leading to the appearance of sinuses. The disease is very chronic and may become healed with subsequent calcification, or may cause death by direct spread. Metastatic spread is rare and constitutional disturbances are slight, pointing to the absence of any considerable degree of to: --

✓ the ✓ and lungs are also attacked. A striking feature of the disease is

dissolved in water, but in older colonies they can be demonstrated either unstained or by the counter-stain in Gram's method. A better method of demonstrating clubs in sections is to stain with hæmatoxylin for 2 minutes, wash, stain with strong carbol fuchsin for 2 minutes, wash and decolorise with alcoholic picric acid for 1 minute.

The first recorded culture was made by Boström. This was of an aerobic organism which is probably a saprophyte living on grains and grasses and, with them, occasionally introduced into the tissues by accident.



FIG. 85 — *Actinomyces bovis* ($\times 200$).

Actinomyces bovis is a partial anaerobe; that is, it grows best in the presence of a small amount of air. Cultures are made by mixing a washed and crushed granule from an uncontaminated lesion with melted glucose agar, cooled to about 50°. After mixing, the inoculated medium is allowed to cool in tubes. After a few days' incubation at 37° the colonies are visible as whitish, irregular specks which are most numerous and best developed in a zone from 0.5 to 1.5 cm. from the surface of the medium. The colonies, which may ultimately grow to a diameter of 2 to 3 mm., are composed of radially arranged, branching filaments. In glucose broth irregular masses occur at the bottom of the tube. In

the absence of involvement of lymph glands. The lesions are more suppurative than in cattle and chronic abscesses with discharging sinuses are produced. Death is not commonly caused, except by direct spread to vital organs or by the addition of another infection.

The organism is probably a mouth saprophyte which, when introduced into the tissues as the result of an accident, has sufficient pathogenicity.

and anugenicity, the organism here described is broken up by some workers into two species—*Actinomyces bovis* which causes actinomycosis in cattle and *Actinomyces israeli* which causes the same disease in man.



FIG. 87.—STREPTOTHRIX IN PUS (X 950)

In tropical countries a chronic type of inflammation, with suppuration and sinus formation, usually in the neighbourhood of the foot (Madura foot), is of fairly common occurrence. In the majority of the cases, in which yellow granules are found, the causative organism is *Actinomyces madurae*, which resembles *Actinomyces bovis*. No clubs are present in the granules. This organism is a strict aerobe.

From the lesions of many cattle suffering from a disease resembling actinomycosis, with the occurrence of granules

supplied with clubs, a small Gram negative, aerobic bacillus has been isolated by Lignières and Spitz. Pure cultures of this bacillus produce, in guinea-pigs and oxen inoculated with it, lesions similar to those of true actinomycosis in which typical granules with clubs were present. It is doubtful if this organism, to which the name *Actinobacillus lignieresii* has been given, ever causes disease in man. A very similar organism, *Actinobacillus actinomycetemcomitans*, is not uncommonly found in lesions caused by *Actinomyces bovis*. It is probably not itself pathogenic.

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act
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Actinomyces Bovis : Colony characteristics :

Raised, nodular, cream coloured
surface, it is a mass of
microscopic nodules
adherent to the surface.

CHAPTER XLV

VIRUSES AND RICKETTSIÆ

The possibility of the existence of organisms so small as to be invisible by ordinary microscopic methods was first suggested by Pasteur during the course of his classical investigations on rabies. Subsequent work showed that, in a variety of other communicable diseases, a causative organism was neither demonstrable by microscopic methods nor capable of being cultivated on laboratory media. In many of these conditions, however, it was found possible to transmit the disease to susceptible animals by the inoculation of filtrates of organs. Although it was suggested by some workers that the infectivity of these filtrates might be due to some property of the fluid, a *fluidum contagium*, it has now been clearly shown to be due to very small, particulate, living agents. These agents are known as viruses.

Viruses differ from bacteria in two important respects: (1) they are very much smaller and (2) they cannot be cultivated on inanimate laboratory media. In many of their characteristics, however, they resemble bacteria—in their ability to multiply in the tissues of the host and to produce disease therein, in their mode of transfer from host to host, in their capacity to stimulate the production of antibodies and in their susceptibility to variation. Thus, whatever the ultimate biological status of the virus particle, virus diseases can, from the practical point of view, be considered as essentially similar to diseases caused by bacteria and can be studied by essentially similar methods.

The sizes of some of the more important viruses are shown in the accompanying table where they are compared with staphylococci, rickettsiæ and serum albumin and globulin molecules. As will be seen, there is a gradation in size from the comparatively large viruses of the psittacosis group to small viruses such as that

of poliomyelitis, the size of which is only slightly greater than that of a protein molecule.

As a result of their small size, it is difficult or, in some cases impossible, to see viruses with the ordinary microscope. Using direct illumination by visible light, the limit of resolution of the microscope is about 0.25μ . Below this size, particles may be visible though their morphology cannot be determined. Theoretically the limit of visibility with direct illumination is about 0.07μ and with the dark ground microscope even less. Consequently, dark ground illumination is widely used in the examination of



FIG. 88—ELEMENTARY BODIES OF A VIRUS (ECTROMELIA), CAUSING DISEASE IN MICE, AS SEEN WITH DARK GROUND ILLUMINATION ($\times 600$)

viruses: with it photographs have been taken of particles with a mean diameter of as little as 0.025μ . By staining methods, which increase the apparent size of the particle through deposition of dye on its surface, viruses can frequently be rendered more readily visible.

Particles of less than 0.25μ in diameter can be resolved by the use of rays of shorter wave length than that of visible light, thus, in recent years, considerable information has been obtained by the use of ultra-violet light. Since, however, the retina is not sensitive to the ultra-violet range, the image of the object must be

recorded photographically. The most important recent advance in virus microscopy has been the introduction of the electron microscope.

This instrument has provided evidence of the existence, in some viruses, of an organised structure. Both ultra-violet and electron microscopy have been of considerable value in determining the size of viruses. With the development of these modern methods, it is obvious that the term ultra-microscopic, formerly applied to viruses, must be considered obsolete.

A second consequence of the small size of viruses is their ability to pass through filters which retain bacteria, a fact which, under which filtration takes place, the term filterable is, however, an indefinite one and unsuitable as a basis of classification.

Since filtration depends on the factors determining the rate of passage through a filter, it is the relation of the diameter of the particle to the pore size of the filter. Filtration is, however, influenced by a number of other factors.

It has been shown that viruses are electrically charged; they will, therefore, tend to be absorbed on to filters which carry an opposite charge. During filtration they may also be adsorbed on to proteins and particles present in the suspension. Other important factors influencing the filtration process are the pressure employed, the time taken and the composition of the fluid in which the material is suspended. Collodion filters or gradocol membranes which have much less tendency to adsorb viruses than earthenware filters and which can be prepared in different grades of porosity, have been of special value in the determination of virus size.
 ✓ Filtration by gradocol membranes is known as ultra-filtration.
 In addition to microscopy and ultra-filtration, the size of virus

particles can be determined by measuring the rate of sedimentation under rapid centrifugation. This method involves the use of elaborate and expensive centrifuges capable of rotating at speeds of 60,000 r.p.m. or more.

✓ So far, none of the pathogenic viruses has been cultivated on the inanimate laboratory media used for the growth of bacteria.

✓ A considerable number, however, can be grown outside the

virus culture has been the introduction of the developing chick embryo as a culture medium. Inoculation may be onto the chorio-allantoic membrane, into the allantoic or amniotic sacs, into the yolk sac or even into the embryo itself. The method of inoculation used will depend on the nature of the virus and on the purpose of the inoculation

It has been suggested that the pathogenic bacteria

trace a gradual loss of synthetic ability in bacteria as we pass from the less to the more highly pathogenic types; it is, therefore, possible that viruses are forms of life in which this process has been carried to a stage where the organism is almost or completely devoid of synthetic power. On this view, the plant viruses, such as the bushy stunt tomato virus, which have been isolated in crystalline form, as nucleoprotein molecules, represent the final stage to which this evolution or devolution has progressed.

It must be borne in mind that our failure to cultivate viruses on inanimate media may be a result of our ignorance of their nutritional requirements and of the optimal conditions of their growth. Another point to be remembered is that the final criterion of the presence of virus is the production of lesions in susceptible animals; it is, therefore, possible that saprophytic

viruses exist which may be capable of growth in the absence of living cells.

Like bacteria, viruses show considerable differences in their susceptibility to physical and chemical agents. As a rule, they are destroyed by heat at 55°-60° for half an hour. They are, on the whole, less susceptible than bacteria to chemical agencies. This chemical resistance is most marked in relation to glycerol; many viruses can survive for long periods in 80 per cent. glycerol which is fatal to practically all non-sporing bacteria. In fact, many viruses survive better in glycerol at low temperatures than in any other suspending fluid. The low susceptibility to glycerol is made use of in the preservation of vaccinia virus in calf lymph for prophylactic immunisation against smallpox. Some viruses, however, are less resistant to glycerol than the majority of bacteria. Although a few of the larger viruses are susceptible to the action of the sulphonamides, the majority are not. Several of the larger viruses, (e.g. those of psittacosis and of lymphogranuloma venereum) are sensitive both to penicillin and to chloromycetin. In their mode of spread, diseases caused by viruses are essentially similar to those caused by bacteria. As in bacterial diseases, the source of infection may be human or animal cases, convalescents or carriers.

Some virus diseases, e.g. herpes febrilis, appear to develop as the result of the activation of a latent virus present in the tissues of an otherwise healthy individual. In the case of herpes, this activation may be due to some superimposed disease such as pneumonia. The presence of latent virus in experimental animals may give rise to considerable confusion in the interpretation of inoculation experiments. In some instances a virus latent in an animal has been aroused by inoculation and has been falsely regarded as the causative agent of disease in the individual from which the inoculated material was obtained.

Though viruses are capable of growth only in the presence of living cells, they are present in secretions and tissue fluids in a viable, but non-multiplying, state. These fluids constitute, therefore, a vehicle, as in the case of bacteria, whereby virus may be

transferred from one individual to another. ✓ The nasopharynx is the commonest portal of entry. Infection may also occur by contact, as in herpes genitalis and labialis, by wound contamination, as in rabies, or through the intermediary of an insect vector, as in yellow fever.

Many viruses show a specificity for one particular animal species, but others are capable of infecting a number of different species. ✓ Certain human diseases are due to viruses primarily causing

through rabbits, loses most of its pathogenicity for man and for dog.

Certain viruses appear to produce lesions mainly in one particular tissue, this specific tissue affinity is called tropism. Some, such as the rabies and poliomyelitis viruses, primarily affect nerve tissues, while others, such as the varicella and herpes viruses, primarily affect the skin. On the other hand, some viruses, such as that of dengue, cause a generalised infection and localisation is an insignificant feature of the disease. ✓ The tissue specificity of viruses is not absolute as, even though the lesions may occur predominantly in one particular tissue, the virus is frequently present in other tissues and specific symptoms are not infrequently associated with a general systemic disturbance.

Some virus diseases show a degree of infectivity much greater than that shown by the majority of bacterial diseases the high infectivity of influenza, measles and variola is widely recognised, on the other hand, some virus diseases have only a low grade of infectivity.

In many virus diseases, structures known as inclusion bodies ✓

These structures, which are in the nucleus, frequently appear. Cytoplasmicophilic. ✓ True inclusion

bodies appear to represent intracellular colonies of virus: in some the virus particles may be seen in the substance of the inclusion as minute bodies, usually of basophilic nature. In many diseases, the virus particles or, as they are called, the elementary bodies, may be distinguished lying free in the cell cytoplasm or in tissue exudates. The demonstration of typical inclusion bodies and elementary bodies plays a considerable part in the diagnosis of certain virus diseases. Considerable skill may be required, however, to distinguish them from non-specific inclusions present in cells not invaded by virus.

The essential changes produced by the growth of viruses in cells are of two types: (a) hyperplasia, as in variola or the common wart: (b) necrosis, as in yellow fever and poliomyelitis. An inflammatory reaction is found in many cases. In some conditions, such as lymphogranuloma inguinale, the cells are predominantly polymorphonuclear leucocytes, in others they are predominantly lymphocytic in type. It is not clear whether these inflammatory changes are a direct result of the presence of virus or are produced secondarily in response to the primary hyperplastic or necrotic effects of the virus on the tissue cells.

As in the case of bacteria, antibodies can be demonstrated in the sera of animals suffering from experimental or natural virus infections. These antibodies are similar in character to antibacterial antibodies and may be demonstrated in vitro by such techniques as agglutination, complement fixation and flocculation. It must be borne in mind that, as but few viruses produce toxins, the antibodies are directed mainly against antigens present in the virus. By means of these antibodies, the antigenic structure of a number of viruses has been elucidated. Like bacterial antigens, virus antigens are frequently highly specific, but in some cases anti-

into a susceptible animal, no lesions are produced. Protection tests, based on this phenomenon, are widely used in the estimation of the antibody content of antiviral sera. In carrying out serological

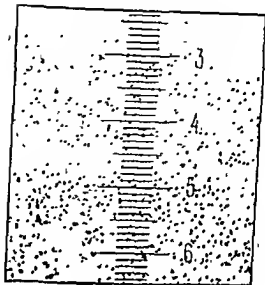
tests with viruses, special precautions have to be taken to eliminate errors arising from non-specific reactions. Such errors are particularly liable to occur since it is not usually possible to obtain virus antigen suspensions free from non-specific materials derived from the tissue in which the virus has been grown.

Antiviral antibody plays an essential part in immunity developed in man against virus infection. The immunity mechanisms are substantially the same as those which have been discussed in connection with antibacterial immunity. In some virus diseases—smallpox, poliomyelitis, chickenpox, measles, yellow fever and mumps—this immunity is of lasting type, one attack protecting for life. In others, however, only a transient degree of immunity is produced. Diseases of this latter type are herpes febrilis, influenza, common cold and trachoma and in these repeated attacks may occur.

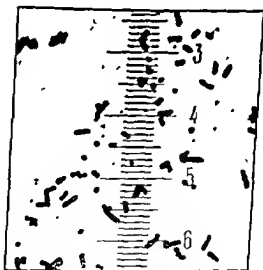
Active immunity in a number of virus diseases may be produced by the inoculation of virus vaccines. These vaccines may be suspensions of killed virus as in the prophylaxis of rabies, or of living virus as in the prophylaxis of variola and yellow fever. If, however, living virus is used, it is only suitable for inoculation in attenuated form. Nowadays, in view of the simplicity of the technique, virus vaccines are obtained, where possible, from growth in the chick embryo. Such vaccines should be administered with care to individuals showing sensitivity to eggs. An alternative method of preparing virus suspension for immunisation is by using extracts of the tissues of infected animals; this is the method used in the various forms of rabies prophylaxis.

In the diagnosis of virus diseases the laboratory can and undoubtedly will in the future play a very important part. In some conditions it is possible to demonstrate virus by direct microscopic methods. In smallpox the elementary bodies can be found in scrapings of papules and vesicles and in trachoma typical

which material from the lesion is tested against immune sera.



A



B

FIG. 89—VIRUSES AND BACTERIA

A—Elementary Bodies of *Vaccinia* Virus from conjunctiva of rabbit; B—*Staphylococci* and *Bact. coli*. Both A and B stained by Paschen's Stam. Both $\times 1200$. Each division on micrometer scale = 1.1μ .

Thus, in cases of variola, extracts of crusts or vesicle fluid give positive flocculation and complement fixation with immune anti-vaccinal serum. Where possible an attempt should be made to isolate the virus by inoculation into the chick embryo or into a susceptible animal. In either case the presence of virus may be inferred from the development of characteristic lesions and in some cases the presence of typical inclusion bodies. Influenza virus is of interest in that amniotic or allantoic fluid of an infected egg will cause agglutination of red cells from a variety of animal species and this capacity may be utilised in determining the

TABLE OF SIZES

	SIZE IN μ
Staphylococcus . . .	1000
Rickettsia . . .	300-1000
✓ VIRUSES	
Psittacosis . . .	350
Variola and vaccinia . . .	250
Herpes febrilis . . .	125
Rabies . . .	125
Influenza . . .	100
Staphylococcus phage . . .	50
Equine encephalomyelitis . . .	30
✓ Yellow Fever . . .	18
✓ Poliomyelitis . . .	10
MOLECULES	
Serum globulin . . .	63
Serum albumin . . .	56

presence of virus. In many cases, information of value may be obtained by estimation of the antibody content of the patient's serum; the most useful technique for this estimation appears to be that of the complement fixation test.

Rabies

Rabies or hydrophobia is a disease affecting practically all mammals, but occurring with greatest frequency in dogs and wolves. Infection is conveyed by the saliva of a rabid animal coming in contact with a cut or abrasion of the skin, most usually as the result of biting.

In man there may be, after an incubation period of from 15 days to 2 months (rarely up to 7 months), a prodromal pain in

the region of the wound and considerable depression. Breathing becomes difficult and swallowing is painful, owing to convulsive contractions of the muscles of the throat. This is frequently an outstanding symptom and the fear of bringing on a spasm may be so intense that even the thought of swallowing anything, not merely water, may bring on an attack. It was this that gave rise

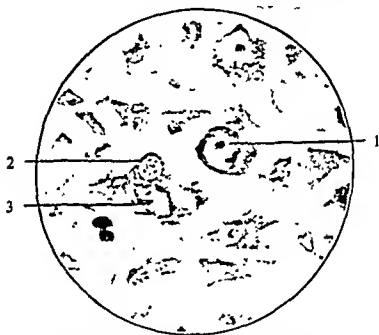


FIG. 90.—NEGRI BODY IN NERVE CELL ($\times 850$).

1. Nucleus of nerve cell.
2. Negri body showing a rosette-like arrangement of its granules around a central structure
3. Degenerated nucleus of nerve cell.

to the belief that the victim feared water, and hence the name hydrophobia. Spasms become general in all parts of the body and all reflexes are increased. The temperature is elevated, the pulse rapid, and delirium is frequently present. Later, weakness comes on and, after a short paralytic stage, death occurs, usually after from 3 to 7 days' illness.

The post-mortem evidence of the disease is not striking. There

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They vary from 0.25μ to 20.0μ in diameter. They may be round, oval or irregular in shape, depending on their position in the nerve cell. Their outline is sharp and definite. In general they are eosinophilic in staining reaction, but frequently basophilic granules may be seen in their interior.

The virus is from 100 to $150\text{ m}\mu$ in diameter and is capable of passing through a Berkefeld and the coarser Chamberland filters. It can be grown in tissue cultures of embryo brain and also in the developing chick embryo. The virus is killed in 1 hour at 50° and in less than half an hour at 60° . Exposure to 5 per cent. phenol kills it in less than an hour. It dies in a few days as a result of drying.

The virus affects chiefly the central nervous system, but is also present in the salivary glands and so in the saliva. As has been proved in a number of cases, the saliva is infective several days before the appearance of the first signs of the disease. In the natural disease in man, infection is almost always from the bite of a dog, wolf, or other rabid or pre-rabid animal. Only about 16 per cent. of those bitten by such an animal and untreated acquire rabies. The incidence appears to depend chiefly on the severity and depth of the bite, its locality and the amount of saliva which comes in contact with the wound. Bites on the face and hands are most dangerous, while those through clothing are least. The influence of locality, apart from such accidental circumstances as the amount of clothing encountered, depends chiefly on the nerve supply of the part. A region richly endowed with nerves is the

most favourable for the development of the disease, for the virus, like the toxin of tetanus, passes up the nerves to the cord and thence to the brain.

Pasteur discovered that the virulence of the virus could be modified by passage through a series of animals. The virus found in the nervous system of the naturally infected dog of the Paris streets, usually known as "Street Virus", had an incubation period when injected subdurally in rabbits of about 12 to 14 days. After passage through a series of rabbits its incubation period was reduced and it caused paresis on the sixth or seventh day in rabbits, but it had then lost most of its pathogenicity for the dog and also for man. The virus, as a result of passage, acquired strongly neurotropic properties and became non-pathogenic to the rabbit when inoculated subcutaneously. Further passage did not increase its virulence for rabbits, and so it was known as "Virus Fixe". Pasteur found that by drying the spinal cord of a rabbit infected with virus fixe he apparently could reduce still further the virulence of its contained virus. A spinal cord dried for 6 to 8 days was of very slight virulence, while 14 days of drying completely deprived it of all its pathogenicity. As a result of his animal experiments, Pasteur was led to try to produce an artificial immunity in a boy bitten by a rabid dog. Hope was given by the fact that immunity could be produced rapidly in animals, while in man the natural disease had a long incubation period. If the immunising process was completed before the elapse of the period of incubation, the disease would not appear. Pasteur's first test was successful, and many years of brilliant successes with many thousands of lives saved have rendered testimony to the essential correctness of Pasteur's theories. The process cannot be regarded as therapeutic, but rather prophylactic; active immunity must be produced before the disease develops itself.

In the original Pasteur method the patient received, daily or twice daily, a subcutaneous inoculation of a suspension, in saline, of about 1.0 cm. of a cord taken from a rabbit dying with virus fixe

infection. For earlier injections, cords which had been dried over caustic potash for 14 days were used and, in succeeding injections, cords dried for shorter times until, for the last injection, from 14 to 21 days after the commencement of the course, a cord dried for only 1 day was employed. Pasteur believed that the drying process reduced the virulence of the virus. Actually it appears to cause the death of virus, very little, if any, remaining alive after 10 days' drying. Although Pasteur's original method of inoculation is still used in some institutes, other and more convenient methods are now available. Killed virus appears to be as effective as is virus prepared by Pasteur's method. Paralysis may follow the use of either living or killed vaccine, but occurs less frequently with the latter.

The serum of animals immunised against rabies has been used in some institutes. It is, however, of little value in some institutes.

As regards the prophylaxis of rabies, the most important step is to abolish rabies in dogs. That this can be done by quarantining all imported dogs and enforcing muzzling, when the disease breaks out, is shown by the great success of these measures in keeping Great Britain and Ireland free of the disease for many years. Thorough cauterisation of bites, either with the actual cautery or with fuming nitric acid, if applied within a short time, although even at the end of several hours it is not without effect, very greatly reduces the risk of contracting the disease. All those bitten by proved rabid animals should be treated with anti-rabies vaccine.

Diagnosis of the disease in dogs is of great importance as a means of deciding whether a human being, bitten by a suspected animal, should receive prophylactic treatment. If the dog has not developed paralysis, it should be kept alive until this occurs, as it invariably does in rabies. Diagnosis may be made by the finding of Negri bodies in the brain of a dog killed in the paralytic stage. If the dog has been killed before paralysis occurs, portions of Ammon's horn should be inoculated intracerebrally into mice

Some of the mice are killed at intervals and their brains are examined for Negri bodies. Others are allowed to survive and are observed for signs of rabies.

Poliomyelitis

This is a disease of childhood, but adults are not entirely immune. It occurs chiefly in sporadic form, but from time to time epidemics, which may assume considerable proportions, occur. The onset of the disease is usually abrupt with pyrexia, frequently accompanied by sore throat. In a few days weakness of a group of muscles, usually in a limb, is observed and this may advance to paralysis. The mortality is low but, as a result of the disease, a considerable degree of deformity is produced, owing to paralysis, although the extent of this, after recovery from the disease, is not so great as in the acute stage. As in meningococcal meningitis, more persons harbour the virus without demonstrable evidence of poliomyelitis than actually develop the disease. Certain factors, and especially physical exertion, appear to predispose to its development.

The pathological change, which may be present in any part of the central nervous system but is of most frequent occurrence in the cervical and lumbar regions of the cord, is chiefly an acute inflammation with hyperæmia and an exudation of mononuclear leucocytes into the perivascular lymphatic spaces. Thrombosis or rupture of small vessels is commonly present. The changes are most marked in the anterior commissure and in the anterior horns of grey matter. As a result, some of the nerve cells die with subsequent degeneration of the motor fibres. The meninges, posterior ganglia and posterior cornua, while similarly affected during the acute stage, undergo little permanent damage. In the cerebro-spinal fluid, a moderate number of polymorphonuclear leucocytes may be found at the onset, being later replaced by mononuclear cells.

The virus, which is very minute (8 to 12 μ), is capable of passing through Chamberland and Berkefeld filters. It is readily killed by heating, a temperature of 45° to 55° destroying it in less

than half an hour, but it resists drying and cold well, and may survive for more than a month at -2° . It is easily killed by potassium permanganate and hydrogen peroxide. The virus is present, in greatest concentration, in the central nervous system, but has also been found in the salivary glands, the mucous secretions, the saliva, nasal secretions. It has not been found in the cerebro-spinal fluid, and but rarely in the blood and solid organs apart from the brain.

The virus, in material from the human body, is transmissible only to monkeys, notably *Macacus rhesus*. The virus has not been cultivated in the developing chick embryo.

The disease is spread by cases, by convalescents and by healthy carriers. There has been considerable dispute as to whether infection occurs by the respiratory or alimentary routes. Experimentally it is possible to produce the disease in monkeys by swabbing the nasal and naso-pharyngeal mucosa and cases have been described in which droplet infection was the only likely method of transfer. On the other hand, the virus can more frequently be recovered from the alimentary than from the respiratory tract. In its geographical and seasonal distribution (rural rather than urban and summer and autumn rather than winter and spring) it more closely resembles enteric fever than cerebro-spinal fever, suggesting alimentary rather than respiratory spread. It is possible that both routes are involved. Although the virus has been found in flies, it is not clear that they play a part in the spread of the disease. The virus appears to gain access to the central nervous system by way of the cranial nerves and those of the sympathetic and para-sympathetic systems and not by the olfactory bulbs as was at one time believed.

One attack of the disease appears to give almost complete immunity for life. Second attacks have very rarely been described. The serum of a recovered case is capable of neutralising the virus and convalescent serum has been widely used during epidemics in an attempt to confer passive immunity. There is no evidence that this has been of any value. Attempts to produce active immunity

with killed virus have been unsuccessful and the use of living virus, even after its virulence has been reduced, has proved dangerous. The most important prophylactic measure is the isolation of cases and contacts.

Encephalitis

There are a number of diseases of the central nervous system presenting ... are presumed or have been ... among these the most familiar ... No specific virus has yet been proved to be the causative agent of this condition although a number of workers have claimed to have isolated such a virus by the inoculation of rabbits. Such claims must be accepted with considerable reserve for two reasons. The first is that spontaneous encephalitis is by no means rare in rabbits. The second is that the virus of herpes febrilis, which causes encephalitis in rabbits, has been found in the cerebro-spinal fluid of normal persons and might, therefore, be present in the fluid of persons suffering from encephalitis lethargica without having any causal connection with that disease.

Other viruses, which are believed to be specific, have been isolated from cases of other types of encephalitis including St. Louis and Japanese encephalitis. The virus of equine encephalo-

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suggest tuberculous meningitis, follow a short illness resembling

fatal cases are rare. The causative virus, which is about 50m μ in

suggestive. Blood and cerebro-spinal fluid should be used for
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Herpes

from a case of herpes febrilis, a keratitis is produced and from this the disease may be transferred again to man. In the rabbit also, the fluid, injected subdurally, produces meningo-encephalitis pathologically similar to that found in human encephalitis. The same condition may result from corneal inoculation with a highly virulent strain. The causative virus is probably widely distributed throughout the body in man: inoculation of rabbits with the cerebro-spinal fluid of a human case produces typical lesions.

✓ The virus can be cultivated in a tissue culture of the cells of rabbit testis and on the chorio-allantoic membrane of the developing chick. The cultured virus gives rise to the same lesions in the rabbit and man as does human herpes material.

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individual may suffer from repeated attacks of herpes although his serum may possess a large amount of neutralising antibody. It is probable that the virus remains in the body between the attacks and is liable to be stirred into activity by intercurrent infection.

Herpes genitalis is characterised by the occurrence of vesicles on the penile and vaginal mucosa. It is usually contracted during sexual intercourse. The virus of this type of herpes is less pathogenic for the rabbit than is that of herpes febrilis and appears to be a distinct, though similar, virus.

Herpes zoster, a disease affecting chiefly adults, is characterised by the occurrence of crops of papules which become vesicles on the trunk or, less commonly, the head. The distribution of the

and, in children, may produce that disease.

Variola and Vaccinia

Variola or smallpox occurs in two forms—the classical type, variola major, and a mild type, variola minor or alastrim. Infection in both types usually occurs through the respiratory route by the inhalation of infective material. The material may be droplets from the respiratory tract of a patient, dust containing dried droplets or matter derived from the skin lesions of a patient. Less frequently the infective material may be introduced into the body through a wound of the skin. Somewhat similar diseases, known as animal poxes, occur in various species of animals.

The viruses of the two forms of variola, of vaccinia and of some of the animal poxes are similar and are closely related to one another, but are not identical.

Variola virus is widely distributed throughout the body in the disease: its presence has been demonstrated in the blood. In the skin lesions, cytoplasmic inclusions, known as Guarnieri bodies,

may be found at some stage of the disease. These bodies, which are spherical in shape and acidophilic in reaction, are comparatively large, measuring up to 10μ in diameter. Although usually homogeneous, they may appear distinctly granular. In addition to the Guarnieri bodies, these are actual variola virus particles.

The virus can be grown in tissue culture and on the chick chorio-allantoic membrane.

The close relationship between the viruses of variola and vaccinia is demonstrated by the possibility of transforming the variola virus into a virus indistinguishable in its effects from the vaccinia virus and by the cross immunity which exists between the two diseases. By passage through calves and rabbits, the virus of variola assumes the properties of the vaccinia virus and, when reinoculated into man, gives rise to vaccinia and not to variola. Repeated man to man passage does not cause the virus to regain its capacity to produce smallpox. Monkeys can be completely protected against variola by inoculation with material containing vaccinia virus and, conversely, against vaccinia by inoculation with material containing variola virus. There can be little doubt that both variola and vaccinia viruses were originally derived from the virus of variola, from the virus of vaccinia or from a common ancestor of the two.

Immunity against variola can be obtained by his inoculation is known as vaccination. The protective effect of vaccinia was demonstrated in 1796 by Jenner who observed that milkmaids, who had suffered from cowpox as a result of milking infected cows, were immune to smallpox. He inoculated a boy with cowpox material and later attempted to inoculate him with smallpox, but without effect. As a result of this and further experiments, he became convinced that an attack of one disease conferred immunity against the other. Vaccination has been widely practised and is, by every competent observer, acknowledged to be one of the greatest advances in medical science. The immunity to small-

pox conferred by vaccination is not complete for life, and re-vaccination

years later.

exceedingly

vaccination in infancy appears to furnish some protection throughout life as, in such persons, if smallpox does develop, it is of mild type and rarely fatal. Formerly vaccination was frequently done with material taken from the pustule of another child. To this arm-to-arm vaccination there were serious objections, and now "calf lymph" is used. This is prepared by wide vaccination on the abdominal skin of a healthy calf, very stringent precautions as to cleanliness being taken. After 5 or 6 days the affected area is thoroughly washed, any crusts are removed and the vesicles are curetted off. The pulpy mass so obtained is ground in 50 to 80

at present, is passed bacteriological tests it is issued for use. Recently vaccines prepared from the virus grown in a tissue culture of chick embryo or on the chorio-allantoic membrane have been used for vaccination. It appears doubtful if the immunity produced is as effective as that resulting from vaccination with calf lymph.

Occasionally, in an adult who has been vaccinated, encephalitis or encephalo-myelitis develops. Vaccination rarely occurs after vaccination in the first year of life. Its incidence has been estimated at 1 in 50,000 vaccinations. The fatality rate is about 60 per cent. The cause of this post-vaccinal encephalitis is unknown, the chief theories being that it is due to vaccinia virus;

used; or present in

develops

as a result of vaccination. This condition, which is a grave one, has a fatality rate of 30 to 40 per cent. Such complications of vaccination as tetanus and suppuration can be completely avoided

by proper precautions in the preparation of the calf lymph and by aseptic technique in vaccination.

/ In countries where vaccination is practised, the clinical diagnosis of smallpox may be very difficult, since such cases as occur are usually mild and atypical. Considerable advances have, however, been made in recent years in the laboratory diagnosis of the disease. There is, unfortunately, no laboratory method which will give any information of value in the initial febrile stage, prior to



FIG 91—VACCINIA VIRUS
(Electron microscope)

the development of the rash. When skin lesions have developed, scrapings from these may be examined microscopically for the presence of elementary bodies. When pustulation occurs, these bodies become less numerous and may be confused with debris. It is not possible to differentiate microscopically the elementary bodies of variola and vaccinia and since, in either disease, elementary bodies may be very few, failure to identify any does not exclude a diagnosis of smallpox. Microscopic examination should be supplemented by an attempt to isolate and identify the virus.

This is best done by inoculation of infected material on the chick which are vaccinia inoculation of material obtained from papules, vesicles, pustules or crusts. Paul's test, in which the cornea of a rabbit is scarified with a scalpel which has been used to scrape variolous skin lesions, is an alternative but less satisfactory method of isolation. Diagnosis is made by histological demonstration of Guarnieri bodies in the excised cornea. Precipitation and complement fixation tests in which crusts or the contents of vesicles and pustules are tested with antivaccinial serum are of value; these tests will not, of course, distinguish between variola and vaccinia.

The patient's serum may be examined for antibody against a vaccinia antigen prepared from lesions produced in rabbits or a variola antigen prepared from smallpox crusts. The complement fixation technique is usually employed. These tests are not generally of value until after the first week of the disease. Individuals who have been vaccinated within a few months prior to the test also give a positive result.

Varicella

Epidemiological and immunological evidence indicates a close similarity between the virus of herpes zoster and that of varicella. The varicella virus has not, however, been grown outside the body either in tissue culture or in chick embryo. It is present in fluid taken from vesicles. Acidophilic intranuclear inclusion bodies are present in affected epithelial cells. It is generally accepted that the human being is the only species capable of being infected with varicella. The serum of a patient agglutinates a formalised suspension of elementary bodies obtained from vesicular fluid.

Yellow Fever

This is a disease, generally with a high fatality rate, formerly common in endemic and epidemic form in tropical and sub-

tropical parts of America and Africa. The outstanding features of the disease are high temperature, great prostration, vomiting, hæmorrhages and jaundice. Infection is conveyed from man to man by mosquitoes, particularly *Aedes ægypti* (*argenteus*), formerly known as *Stegomyia fasciata*. Infection is only possible if the insect has fed from a patient in the first three days of the disease, but the insect does not become infective to another

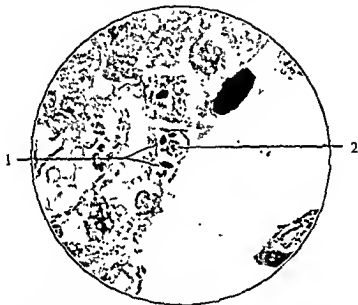


FIG 92 —YELLOW FEVER INCLUSION BODY (HUMAN LIVER) (X 1000).

1. Peripheral masses of chromatin
2. Irregular granular ring-form of Inclusion Body surrounding a central mass of chromatin

individual for at least 4 days, and not for a much longer time if air temperature is low. Infection may be conveyed directly from man to man by contact with the blood or serum of a patient.

When the mode of transfer of the disease from man to man by *Aedes ægypti* was discovered, it was hoped that the disease would be rapidly stamped out by control of the mosquito. This measure has been very effective in dealing with the form of the disease and forming a dense acidophilic mass in the cytoplasm.

carried by *Aedes aegypti*. Another variety of yellow fever, apparently carried by some other species of mosquito, is still widely distributed in Central and South America and Central Africa. In this variety, which is known as jungle yellow fever, the virus is present in the blood of animals of various species. The animals, which are little affected by the presence of the virus in their blood, constitute the reservoir of infection. The virus is transferred from animal to animal, and occasionally to man, by mosquitoes.

Investigation of the disease was hampered by the fact that the majority of laboratory animals are insusceptible but, in Lagos in 1927, it was discovered by Stokes and other members of a Rockefeller Commission that a monkey, *Macacus rhesus*, could be infected. The virus was carried through many monkeys, either by direct passage or through the intermediary of the mosquito. Later mice and guinea-pigs were found to be susceptible to infection by intracerebral inoculation.

The virus becomes neurotropic and loses its viscerotropic character when passaged through a series of mice intracerebrally. It has then lost much of its virulence for man, and a suspension of it, grown in the chick embryo, is now widely used in the living condition for active immunisation of human beings. A dead vaccine has no protective value. Passive immunity can be produced by convalescent serum which is actively antiviral.

Since the serum of a person who has recovered from yellow fever continues to contain antiviral bodies for years, the extent to which the disease is or was formerly prevalent in a community may be ascertained by testing the sera of a number of persons for antiviral properties. This is done by the injection of a mouse with serum and mouse-brain virus. If the mouse survives, the serum contains protective substances indicating recovery from yellow fever. In this way "silent areas" have been discovered, where no typical cases of yellow fever had been known to occur, but where the disease either exists in atypical form or has previously existed.

In the liver cells both of man and of experimental animals, very characteristic bodies, known as Councilman bodies, are found as well as intranuclear inclusion bodies.

It is not inappropriate to record that knowledge of the facts concerning Yellow fever has been obtained only by sacrifice: Lazear in America in 1900, and Stokes, Noguchi and Young in West Africa in 1927 died of yellow fever when investigating the disease.

Influenza

There is now no doubt that influenza is due to a virus and not to H. influenza, the bacterium which was for many years accepted as causative of the disease. The first isolation of the virus was effected in England by Smith, Andrewes and Laidlaw whose technique was the 'instillation of nasopharyngeal washings of influenza patients intranasally in ferrets. A similar, but antigenically distinct, virus was later isolated in America by Francis and others using the same technique. These two viruses are now usually called influenza A and influenza B virus. Since neither of these viruses can be isolated from certain cases of influenza, it is probable that there are also other influenza viruses

infection during non-epidemic periods. ✓ The virulence of the virus is subject to fluctuation. In the absence of an epidemic, it is low. The factors responsible for the increase in the virulence of the virus and thus for epidemics of the disease are unknown. ✓ The disease caused by a virus of enhanced virulence is probably the most infectious disease affecting mankind and is certainly the one with the highest mortality. The pandemic of influenza of 1918-19 was responsible for more deaths than the war of 1914-18.

✓ The virus is transmitted by direct contact with secretions from the nose or throat of an infected person.

effects of the virus and such bacteria as H. influenza, pneumococci and Strep. pyogenes.

The virus, which measures from 80 to 120mμ in diameter, can be grown in the chick embryo either from the artificially infected

ferret or direct from the human subject. Primary growth is more successful in the amniotic cavity than it is in the allantoic cavity. When unfiltered nasopharyngeal washings or other materials are used for inoculating the amniotic sac, it is necessary to add antibacterial substances (penicillin or sulphonamides) to restrain the growth of the bacteria invariably present.

The detection of influenza virus in amniotic or allantoic fluid has been greatly facilitated by the finding that these fluids, when the virus is present in them, cause agglutination of the red blood cells of fowl, man and certain other animals. This agglutination, which is produced by killed as well as living virus (either A or B), appears to be due to the adsorption of the actual virus on the surface of the cell.

present in much greater concentration in immune than in normal human serum and, by determining the dilution of a specimen of serum which inhibits agglutination, the concentration of viral antibody may be ascertained.

The immunity which results from an attack of influenza is of very short duration. Active immunity may be produced in man by inoculation of a vaccine containing the virus (or, preferably, the two viruses), grown in the chick embryo. There is good evidence that the resulting active immunity is sufficient, in many cases, either to prevent an attack of the disease following exposure to infection or at least to modify the severity of the attack. Since, however, this artificially stimulated active immunity does not persist longer than immunity resulting from an actual attack, this method of immunisation is only likely to be of practical value when an epidemic can be predicted and when the predicted epidemic occurs within a few months of the injection of the vaccine.

Diagnosis of influenza may be confirmed by determining the presence of the virus in nasopharyngeal washings, either by intranasal inoculation of a ferret or by inoculation into the amniotic sac of the chick embryo.

A retrospective diagnosis may occasionally be made by determining, by hæmagglutination inhibition or complement fixation, the antibody titres in samples of the serum of a person collected in the acute stage of the disease and during convalescence. A considerable increase in the titre of the second specimen, as compared with the first, suggests that the disease from which the patient suffered was influenza

Psittacosis and Ornithosis

Psittacosis is primarily a disease of birds. It was originally described in members of parrot family, but subsequently birds of other species, including fulmar petrels, pigeons and domestic

Psittacosis is a severe disease with a high fatality rate. It has been mistaken for enteric fever, but lung involvement is common.

The virus of psittacosis, which can be grown in the chick embryo, resembles that of lymphogranuloma inguinale in being of large size (250-400m μ), in staining more readily than most viruses and in producing characteristic cell inclusions, which go through a regular sequence of morphological changes. Diagnosis can be effected by the intraperitoneal inoculation into mice of blood taken during the first 4 days of the disease, in cases showing lung involvement, the virus can be isolated at a later stage from the sputum. Infected mice show peritonitis and enlargement of the spleen, elementary bodies can be demonstrated in smears made from the liver and spleen. The presence of antibodies for the virus in the serum of a patient may be detected by the complement fixation reaction. A titre rising in the acute state and falling during convalescence confirms the diagnosis.

Lymphogranuloma venereum

✓ This disease is contracted by sexual intercourse and appears first as a sore in the genital mucosa in either sex. The inguinal

glands, which are involved by lymphatic spread, enlarge to form a bubo and may break down, producing pus. Systemic symptoms may be present.

The virus which, as has already been stated, is a large one, grows in the yolk sac of the chick embryo and cultures may be obtained by inoculating the sac with pus from a bubo. Mice may be infected by intracerebral inoculation.

A diagnostic reaction known as the Frei test antedates the culture of the virus. It consisted of the intradermal injection of the suspected patient with heated pus taken from a bubo of another patient. The development of a papule at the inoculation site was regarded as a positive result and indicated that the subject was suffering from lymphogranuloma inguinale. In the test, as now carried out, the material injected is prepared from chick embryo or mouse brain infected with the virus. Antibodies to the virus in the serum of a patient suffering from the disease may be demonstrated by complement fixation using, as antigen, material taken either from the infected chick embryo or mouse brain.

Since there is considerable antigenic overlap in the viruses of lymphogranuloma venereum and of psittacosis, material containing the virus of one disease may give a positive Frei or complement fixation test in the case of a patient suffering from the other disease. For this reason, the results of these tests should be considered critically in the light of the clinical features of the case.

Primary atypical pneumonia

In recent years considerable attention has been given to cases of primary pneumonia apparently not due to bacterial infection. The disease, which is of mild type, is characterised by widespread lung infiltration as judged by radiological evidence. The condition appears to be due to the effects of a virus. In some cases the viruses of psittacosis, lymphogranuloma inguinale, lymphocytic choriomeningitis, Q fever and influenza have been demonstrated, but in others either no virus has been found or the virus isolated has not been completely identified as the causal agent. In some cases of atypical pneumonia, the serum of the patient contains

"cold agglutinins", that is agglutinins which act on human red cells of Group O at 0° but not at body temperature. Such agglutinins are not present in patients suffering from other types of pneumonia, although they occur in the sera of patients suffering from paroxysmal hæmoglobinuria and certain other conditions.

Trachoma

Trachoma appears to be due to a virus similar to those of psittacosis and lymphogranuloma venereum. Characteristic inclusion bodies may be found in the affected conjunctival epithelium. This virus has not been cultivated outside the body.

Measles

There is little doubt that measles is due to a virus. Some workers claim to have grown it both in tissue culture and in the chick chorio-allantoic membrane and state that, after passage in the chick, it becomes attenuated and, on inoculation into children, causes only a modified form of the disease.

Passive protection against measles may be obtained by the injection of a few millilitres of the serum of a convalescent. The immunity developed against measles is a very solid one and the serum of a person who suffered from the disease years before usually contains sufficient antibody to protect, although a larger dose of it may be required than of the serum of a recent convalescent. Since this antibody, like others, is mainly γ globulin, injection of this globulin extracted from pooled, adult blood or from the blood of placentas is now frequently used to confer passive immunity against measles. Injection of serum or globulin in adequate amount soon after exposure to infection with measles usually prevents the development of the disease. If the injection is delayed until about the sixth or seventh day after exposure, the result of the injection is to so modify the disease as to render the

of serum produces.

Hepatitis

... distinguished—
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 ... cholangitis.

Liver tissue obtained by biopsy and post-mortem shows clearly, however, that the disease is a true hepatitis with wide-spread degenerative changes in the liver cells. The disease, which has an incubation period of 20 to 40 days, has been transmitted to human volunteers by oral administration of Seitz filtrates of faeces from early cases. Lack of a susceptible animal and failure to obtain growth in the chick embryo, following the introduction of material believed to contain the virus, have been major obstacles in the study of this form of hepatitis. Infection is probably transmitted by the faeces of a patient to the mouth of another individual.

It is found that a similar condition
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it of immune human serum. It should be noted that the serum or plasma responsible for this condition is always of human origin. The administration of antibacterial and antitoxic sera prepared in horses or other animals does not involve any risk of the development of hepatitis and it is for this reason that the condition is called homologous serum jaundice and not merely serum jaundice. A disease, indistinguishable from this and with the same long incubation period, has occurred so frequently as almost to assume epidemic proportions in the practice of some venereal disease and diabetic clinics. In the former, it has affected patients suffering from syphilis and not those suffering from gonorrhoea. The fact that syphilis is treated with arsenic for a time directed attention to the possibly injurious effects of that metal on the liver, but it was found that while, in one clinic, the incidence of jaundice was very high, in another, using the same drugs, it was

low. It was then realised that the important factor was not the material injected (arsenical preparations, bismuth salts, insulin) but the fact that injections, whether intravenous, intramuscular or subcutaneous, had been given to a number of persons in rapid succession. There is now no reasonable doubt that the hepatitis of clinics is due to the use of faulty technique for cleaning and

the very small amount of blood injected, there is practically no alternative to this theory. The virus of injection hepatitis is probably the same as that of homologous serum jaundice and different from the virus of infective hepatitis.

Other Viruses

Among other human diseases proved or believed to be due to viruses are mumps, common cold, molluscum contagiosum, common wart, German measles, dengue fever, infectious mononucleosis and foot and mouth disease.

The infectious mononucleosis is an important diagnostic test (the

agglutination of sheep red blood cells. Another type of antibody having the same effect is occasionally found in the sera of normal persons and yet another in the sera of persons who have received injections of horse serum. The three antibodies can be distinguished by differential absorption from the serum by a suspension of guinea-pig kidney or of ox red blood cells. The normal antibody is absorbed by guinea-pig kidney but not by ox cells and the horse serum antibody by both. The antibody which is absorbed

Bacteriophage

Bacteriophage or phage is the name given collectively to a large group of viruses which differ from those already mentioned in that they are parasites, not in animal tissue cells, but in bacteria.

Phages show differences in size (from 10 to 90m μ), in morphology as revealed by the electron microscope, in the species of bacterial cells attacked, in the size of their colonies and in resistance to physical and chemical agents.

Phages are obligatory cell parasites and, up to the present, have been cultivated only in living and actively multiplying bacteria. To obtain a culture of a phage we may use a young (4-6 hours) broth culture of a susceptible bacterium. If a minute amount of a preparation of an active phage is added to this and incubation continued, instead of the culture becoming progressively more cloudy, it becomes progressively clearer until it may be as clear as uninoculated broth. At this stage there will be present in the broth a large number of living phage particles and a small number of surviving bacteria. A pure preparation of the phage may be obtained by filtering through a bacteria-proof filter: the filtrate will contain phage free from bacteria. A simpler method occasionally available is based on the fact that, in some cases, the phage is more resistant to heat than the bacterium. By heating the lysed culture sufficiently to kill the bacteria, the phage is left still alive. Preparations of most phages purified by either method can be kept alive in the refrigerator for months.

To identify a phage, we must determine the species or strains of bacteria which it lyses. The presence of lysis may be detected by the clearing of a broth culture of the organism, but only very active phages give complete clearing of such cultures. A more convenient method, which has been described in connection with the phage typing of *Salm. typhi*, is to spread a loopful of a young broth culture of the organism over a small area of an agar plate and to allow the broth to dry and then to deposit a loopful of the phage preparation in the middle of the spread area. After incubation, the presence of the phage will have demonstrated itself by alterations in the bacterial culture. An active phage, present in

large numbers, produces a large, circular, bare area devoid of obvious bacterial growth. Smaller numbers of phage particles cause a moth-eaten appearance in the culture. A still smaller number of phage particles give rise to circular bare areas (plaques) measuring from less than one millimetre up to several millimetres in diameter. Each plaque is a colony of phage which has been grown on the bacteria.

Pure cultures of phages can be obtained from mixtures by serial passage in broth cultures of bacteria susceptible to each type, so securing multiplication, and finally growing each on the sensitive bacteria on agar and cutting out individual plaques.

It appears probable that a single phage penetrates into the interior of a young bacterial cell, multiplies there and causes its disruption, so releasing a crop of new phage particles.

Certain phages show a high degree of specificity as regards their host. Specificity of phage may be not only for a particular species, but also for only those members of the species which have a particular antigen (such as the Vi antigen of *Salmonella typhi*). In a few cases, specificity is carried still further, each phage acting only or best on one of a number of types which, since they all possess the same antigen, cannot be distinguished by serological methods.

Some phages are capable of rapid adaptation, so that they become capable of lysing types of bacteria which are normally resistant to them.

Bacteria are not inert in relation to phage. When a broth culture of a bacterium is acted upon by a phage, apparently complete clearing may occur but this cannot be taken as signifying that every individual bacterium present has been lysed. If the incubation of the culture is continued, turbidity usually redevelops. The bacteria in the secondary growth will be found to be completely or relatively resistant to the phage although they may act as carriers of it.

Apart from observations with the electron microscope, the presence of a phage can be detected only by the effects which it produces on bacteria. Very active phages can be demonstrated by

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of these tumours. Whether a virus plays any part in the causation of malignant tumours in man remains *sub judice*.

Rickettsiæ

Rickettsia constitutes a group of organisms the characteristics of which appear to be intermediate between those of the bacteria and of the viruses. Like viruses, they have not been cultivated on inanimate media, but in size and in non-filterability they resemble bacteria. Although the majority of rickettsiæ proliferate only inside living cells, some (*R. orientalis* and *R. burnetti*) grow in tissue culture in association with, but actually outside, living cells.

The rickettsiæ can be stained by Giemsa's method, appearing

More than forty species have been distinguished, of which only six, *R. prowazekii*, *R. quintana*, *R. rickettsii*, *R. orientalis*, *R. mooseri* and *R. burnetti*, have been shown to be pathogenic for man. These are transmitted to man by the bite of the insect host. The diseases which they produce can be divided into five groups—the Typhus group, the Rocky Mountain Spotted Fever group, Scrub Fever, Q Fever and Trench Fever.

Rickettsiæ are insensitive to penicillin and sulphonamides but Chloromycetin, an antibiotic originally obtained from cultures of *Streptomyces venezuelæ* but now prepared synthetically, inhibits the growth of certain rickettsiæ in the chick embryo and is very effective in the treatment of typhus and tsutsugamushi fevers. Another antibiotic, Aureomycin, produced by *Streptomyces aureofaciens*, is also of value in the treatment of certain of the rickettsial diseases.

Typhus Fever

Two types of typhus fever, classical and murine, can be recognised. Classical typhus, which may occur either in epidemic form

the clearing of broth cultures, less active phages by the production of plaques in agar cultures of the bacteria. Some strains of phage can act as non-pathogenic parasites in bacteria. The bacteria then become carriers of the phage which may be capable of lysing bacteria of another species or type. The presence of a phage in a strain of bacteria, which are not lysed by it, stimulates the development of variants.

At one time it was thought that the administration of phage might be of therapeutic value in the treatment of such diseases as

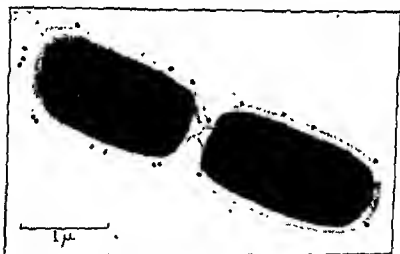


FIG. 93--*Bact. coli* WITH ADSORBED PHAGE PARTICLES.
(Electron microscope)

cholera, *dysentery* and *typhoid fever*. Most reliable observers have now abandoned the hope of phage therapy. The failure of phages in this respect is probably due to the rapid development by bacteria of phage-resistant variants. The most important practical application of phages to-day is for the typing of *Salm. typhi*, *Staph. pyogenes* and other bacteria.

Certain types of new growth, most usually regarded as malignant (e.g. Rous sarcoma), can be initiated by inoculation of a healthy animal with filtrates of extracts of the tumour. It is fairly generally believed that a filterable virus is the cause of production

or from the intestines of lice tediously infected by injection per anum. More recently a vaccine prepared from rickettsiæ grown in the chick embryo yolk sac has been introduced and is now widely used. Vaccines appear either to give protection against typhus or considerably reduce the severity and fatality of the disease.

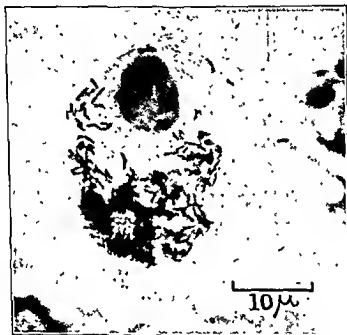


FIG 94 —*R. mooseri* IN ENDOTHELIAL CELL OF GUINEA-PIG TESTIS
(Electron microscope)

Murine typhus is due to *R. mooseri*, an organism closely resembling *R. prowazeki*. The disease is enzootic in wild rats in various countries in which it produces a mild disease. It is transmitted from rat to rat by the rat flea or rat louse and from the rat, which constitutes a reservoir of infection, to man by the rat flea. The serum of the human patient agglutinates *Proteus X*₁₉

or sporadically, is caused by *R. prowazekii* and is spread from case to case by the body louse. A milder form of the disease, which occurs endemically and never gives rise to epidemics, is known as Brill's disease. The rickettsiæ can be demonstrated in the vascular endothelium of the skin, brain and other organs. They can also be found in the lumen and within the epithelial cells of the intestine of lice which have fed on typhus patients. A period of from 2 to 11 days must elapse after a louse has bitten a patient before it becomes infective for another human being. Guinea-pigs can be infected by intraperitoneal inoculation and the rickettsiæ can

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believed that typhus was due to a *Proteus* rather than to a *Rickettsia* and others that the *Rickettsia* was a stage in the development of the *Proteus*, but the view now generally held is that the reaction is due to the presence of the same polysaccharide antigen in the two organisms. This antigen is present in the bodies and not in the flagella of *Proteus* and hence agglutination is of the O type and non-motile strains of *Proteus* should be used for the test. The test is rarely positive in other than rickettsial diseases except in some *Brucella* infections and in patients infected with a *Proteus*. The serum of the typhus patient also agglutinates a suspension of *R. prowazekii*.

✓ One attack of typhus confers immunity against the disease. Some degree of passive immunity may be produced by the injection of the serum of immunised or of the serum of immunised from the injection of vaccines or nearly the necessary suspensions of the organism were obtained from the tissues of infected animals

CHAPTER XLVI

PATHOGENIC PROTOZOA

Malaria

Malaria is a disease chiefly of tropical or sub-tropical countries although it is not unknown in temperate climates and was, until fairly recently, common in certain parts of England and Ireland. It is characterised by attacks of fever which recur at more or less regular intervals for some considerable time. Anæmia and enlargement of the spleen are almost always observable.

The disease is due to the presence in the red blood corpuscles of minute protozoa, the malaria parasites, of which four species are known. These can be distinguished by their microscopic appearance and also by the type of disease produced. Benign tertian malaria is due to the *Plasmodium vivax*; Quartan malaria to the *Plasmodium malariae*; Malignant or Sub-tertian malaria to *Plasmodium falciparum*; another tertian form of the disease is due to *Plasmodium ovale*. Malaria may be conveyed by the injection of blood from a patient into a healthy individual, as has occurred in blood transfusion, but in nature the disease is transmitted exclusively by the bite of a female mosquito of the genus *Anopheles*. In the human body the parasites multiply asexually, while sexual multiplication occurs in the mosquito. For this reason the mosquito is regarded as the definitive host of the protozoon and man as the intermediate host.

The life history of one of the parasites, *Plasmodium malariae*, will first be described, and subsequently the characteristics which distinguish it from those causing the other types of malaria will be mentioned. When the mosquito bites, she injects into the human body a small amount of saliva which may contain a large number of young parasites. Each of these is a minute, thin, pointed rod, having about its centre a nucleus, easily distinguished

and X_2 , the former to a higher titre than the latter, but not X_K . The reaction is thus of no service in distinguishing classical and murine typhus. They can, however, be distinguished by the fact that patient's serum agglutinates a suspension of *R. mooseri* to a higher titre than one of *R. prowazekii*.

Rocky Mountain Spotted Fever Group

This group includes, in addition to Rocky Mountain Spotted Fever, Brazilian Spotted Fever, *Fièvre Boutonneuse*, Kenya Fever and South African Tick Fever. The causative organism of the group is *R. rickettsii* or variants thereof. Rodents, dogs and sheep constitute reservoirs of infection and the diseases are transmitted from them to man by ticks. The serum of patients agglutinates *Proteus* X_{19} and X_2 but not X_K .

Scrub Typhus

Under this heading are included Malayan Scrub Typhus, Tsutsugamushi Fever of Japan and Mite Typhus of the Dutch East Indies which are probably identical diseases. The causative organism is *R. orientalis*. Voles and wild rats are the reservoirs of infection and the transmitting agents are mites. The serum of the patient agglutinates *Proteus* X_K but not X_{19} or X_2 .

Q Fever

Q fever, a disease which occurs in Australia and North America, is caused by *R. burnetii*. The reservoirs of infection are certain wild animals and cattle and the transmitting agent among animals is a tick. In some cases the disease spreads to man by contact with the flesh of infected cattle or by inhalation of dust contaminated with infected tick faeces. The serum of the patient does not agglutinate any of the three varieties of *Proteus* X_{19} , X_2 or X_K .

Trench Fever

Trench fever, which was common in the war of 1914-18, is not now known to exist. It was due to *R. quintana* and was spread by lice. Neither the mammalian reservoir nor the capacity of the patient's serum to agglutinate *Proteus* is known.

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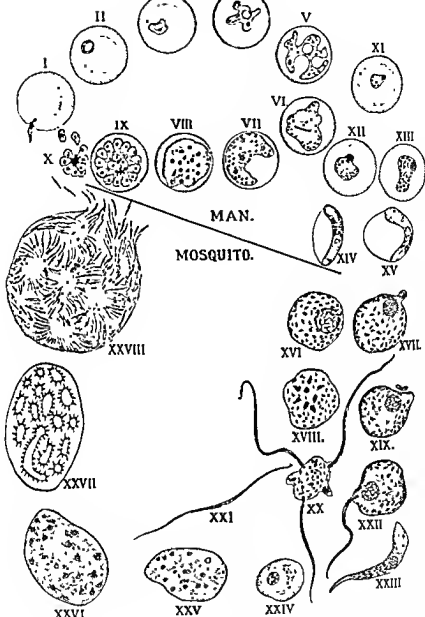
The life history of one of the parasites, *Plasmodium malariae*, will first be described, and subsequently the characteristics which distinguish it from those causing the other types of malaria will be mentioned. When the mosquito bites, she injects into the human body a small amount of saliva which may contain a large number of young parasites. Each of these is a minute, thin, pointed rod, having about its centre a nucleus, easily distinguished

in stained preparations by its chromatin. This, the Sporozoite, does not immediately penetrate a red blood cell as was formerly believed, but first develops in cells of the reticulo-endothelial system, giving rise to young forms which then invade the red blood cells and become Trophozoites. While still young, the trophozoite is commonly found as a ring-shaped body. The ring resembles a signet ring, since it is thicker on one side than the other, and the nucleus is usually situated in the thinner side. The trophozoite grows at the expense of the hæmoglobin of the cell and frequently assumes an oval or band-like shape. Waste material accumulates in its body in the form of coarse grains of a dark brown pigment, scattered chiefly towards its edge, the nucleus now being situated centrally. When examined alive at this stage its outline is found to be constantly altering within the red cell; in fact, it behaves very much like an amœba. The fully mature trophozoite, now the Schizont, almost fills the red cell, which otherwise does not appear to be greatly altered. Various activities take place; the nucleus breaks up into a number of fragments which scatter through the protoplasm, while the pigment accumulates in the centre. After the division of the nucleus the protoplasm also divides, and the parasite is now found to have produced from 6 to 12 new parasites, the Merozoites, which are regularly arranged like the petals of a daisy. A certain amount of residual protoplasm surrounds the central pigment. The red cell disintegrates and the brood of merozoites is free in the plasma and, at the same time, the pigment is also liberated. A merozoite approaches a fresh red cell, penetrates it and develops just as did the original sporozoite, only to divide in course of time into yet more merozoites. The process of multiplication by asexual division in the human body is known as Schizogony. In the case of quartan fever the cycle is one of 72 hours (A paroxysm of fever recurs on the fourth day, counting the day of a bout as one.) If only one sporozoite has been injected by the mosquito it will have produced, say, 10 merozoites at the end of 72 hours; after a further 72 hours there will be 100, and so on. The parasites adhere fairly rigidly to their time-table, so that at any moment the majority are at

exactly the same stage of development, a few being slightly too rapid and a few too slow. The setting free of all the merozoites in the patient's blood, therefore, occurs at about the same time, and it is found that the fever commences at this time. The cause of the fever is certainly some toxic substance, either the pigment or some other waste product, liberated when the red cells disintegrate and the merozoites escape. It has been found that if the serum obtained from the blood of a patient during a paroxysm be filtered and injected into a healthy man it gives rise to a single bout of fever similar to that of malaria.

The disease has an incubation period of about a fortnight, the time necessary for the development of sufficient parasites in the blood. It has been calculated that for the production of symptoms there must be several hundred parasites in each cubic millimetre of blood. From this time onward, in the absence of treatment, bouts of fever occur at intervals of about 72 hours. If, however, the patient has been infected on two occasions, the two broods of parasites resulting from the injected sporozoites will each follow its own time-table, and schizogony of each brood will occur every third day; but the unfortunate patient will experience two bouts of fever every 3 days. In the same way, as the result of three distinct infections, fever may occur every day. Rarely does the mosquito inject a single sporozoite; but however many are injected, the descendants of those injected at the same time all follow the same time-table, the only difference being that a slightly longer time will elapse before the first attack of fever where only one was injected than when the number was large.

Simultaneously with the production of merozoites two other types of cell develop in the patient's blood in preparation for the sexual cycle and sporogony, in the mosquito. The development of these resembles that of the ordinary trophozoite, but no division of the nuclear material occurs. These, the gametocytes, enlarge and become rounded. The Macrogametocyte (female) is larger, takes stain more intensely, and has more pigment in coarse grains arranged in a circle around its centre, while the Microgametocyte (male) is smaller, stains (with Leishman's stain) a grey-blue or



MALARIA PARASITE
 Ixizont. X, Merozoites
 XVII, Macrogameto-
 XXII, Zygote XXIII,
 cyst with Sporoblasts

(Note.—XXVI to XXVIII are shown less highly magnified than the other drawings)

pinkish colour, has less and finer pigment, less regularly distributed, but a larger nucleus. These cells do not develop further in the human body. In the stomach of a mosquito which has swallowed human blood containing the parasites, the macrogametocyte develops into the Macrogamete and the microgametocyte produces a number of Microgametes. One of the latter impregnates a macrogamete which develops into the Zygote. The zygote becomes oval, elongated and, as the Ookinete, almost wormlike. It penetrates the wall of the stomach and attaches itself to the outer surface where it again becomes a rounded body of



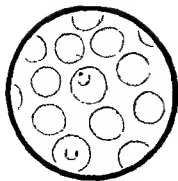
FIG. 96—MACROGAMETOCYTE OF *P. falciparum* ($\times 1200$)

about 6μ in diameter. It grows rapidly, surrounds itself with a capsule and becomes the Oocyst. The oöcyst soon comes to form a large swelling, protruding from the wall of the stomach into the body cavity of the mosquito. The nucleus and protoplasm divide and a number of Sporoblasts are formed, each of which divides into a large number of thin spindle-shaped, nucleated cells, arranged radially. The sporoblasts disappear and these threads, the Sporozoites, are free within the capsule, which may now have a diameter of 60μ . This bursts and the sporozoites find themselves in the insect's body cavity, from which they make their way to the salivary glands and are injected by the insect into the next

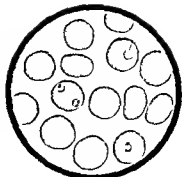
human being on whom she feeds, there to set up the schizogony cycle again. The sporogony cycle occupies on an average from 10 to 12 days.

In *Plasmodium vivax*, schizogony is very similar. The amœboid movement of the trophozoite is, however, much more active. The parasite tends to be rather larger and the red cell is pale and swollen and frequently shows, when stained, deeply stained points, Schüffner's dots. The pigment within the parasite is finer and of a lighter colour. In schizogony from 15 to 20 oval merozoites are formed, which are arranged irregularly. Schizogony occurs every 48 hours. Since it is of a relatively mild type, this form of the disease is called Benign tertian malaria. Sporogony does not differ in essentials from that in the quartan parasite.

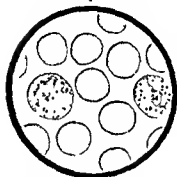
Malignant or Sub-tertian malaria is of a more serious character. The occurrence of the fever is less regular, although mainly every 48 hours, and multiple infections are common. The young trophozoites are smaller and show more active amœboid movements than those of *P. malariae*. When fully developed the schizont occupies less than half the red cell, which appears shrunken and deeper in colour than normal. Pigment is scanty and occurs in fine grains. From 6 to 20 merozoites, irregularly arranged, are produced, but this stage is very rarely seen in the peripheral blood, as the second 24 hours of the cycle, including schizogony, is confined almost exclusively to the capillaries of the internal organs, particularly the spleen. The gametocytes differ from those of the other two parasites since they are of crescentic or sausage shape. They are larger than the cells in which they developed, the remains of which may sometimes be observed as a fine line uniting the points of the crescent like the string in a bow. The macrogametocyte is larger, has pointed ends, stains more intensely, has a smaller nucleus, and the pigment is collected towards the centre. In the microgametocyte, which has rounded ends, the pigment is distributed through the protoplasm of the parasite. Outside the body the gametocytes lose their crescentic outline, become rounded, and further development is very similar to that of *P. malariae*.



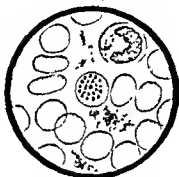
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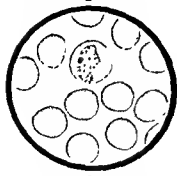
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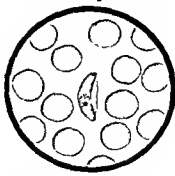
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6

MALARIA PARASITES IN BLOOD FILMS.

1 *P. malariae*, young ring forms.

2 *P. vivax*, trophozoite

3 *P. malariae*, microgametocyte.

4 *P. falciparum*, young ring forms

5. *P. vivax*, schizont

6. *P. falciparum*, macrogametocyte.

human being on whom she feeds, there to set up the schizogony cycle again. The sporogony cycle occupies on an average from 10 to 12 days.

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The laboratory diagnosis of malaria depends on the finding of the parasite in the blood. Three methods are employed: in the first a fresh thin film of blood is examined unstained; the second is to make blood films in the ordinary way and to stain with Leishman's or some other stain; the third, the thick-film method of Ross, is employed where parasites are few. About 20 c mm. of blood are spread over an area of about 1 square inch and allowed to dry. The film is inverted in a few drops of a 1 : 15 dilution of Giemsa stain and left there for 20 minutes. This both lakes the cells and stains the parasites. It is then washed, dried and examined. Some experience is needed before the observer can rely on finding all stages of the parasite when present, and on distinguishing platelets and artefacts, such as deposited stain, from parasites. A prolonged search may be necessary before even a single parasite is found. In the absence of parasites, the presence of an excess of large mononuclear leucocytes in the blood and of pigment in the leucocytes may assist in establishing a diagnosis.

It has been found possible to cultivate the malaria parasite in defibrinated blood containing 0.5 per cent. glucose at a temperature of 40° to 41°. Schizogony proceeds within the red cells, but sporogony does not occur.

The chief symptoms of malaria are due to the toxic products of the parasite which are thrown into the blood stream at schizogony. In addition, anæmia is present, and the number of red cells is diminished to an extent which cannot be explained by the number infected. There appears to be a definite destruction of the cells by hæmolysis, due either to the pigment or other toxic product. Leucopenia is usually to be observed, but there is an increase in the number of large mononuclear cells. The most obvious pathological change is the deposition of the pigment in the internal organs, especially in the spleen, liver, bone marrow and brain. The pigment is found chiefly in the endothelial cells of the capillaries, which, in consequence, may degenerate, leading to capillary hæmorrhages; but in the spleen and bone marrow it is found all through the tissues.

Amœbic Dysentery

Amœbic dysentery is a disease quite distinct from that due to the presence of the dysentery bacilli. The onset is more gradual, there is little fever, and the number of motions passed is few but, as in the bacillary disease, they contain both mucus and blood. In the absence of appropriate treatment there is a marked tendency for the disease to assume a chronic form, the patient becoming more and more emaciated. Sporadic cases of amœbic dysentery are common in tropical and sub-tropical countries; epidemics are rarely experienced.

The organism, the Entamœba histolytica, is found in the feces, in the ulcers of the intestine and in the abscesses of the liver which are a frequent complication of the disease. If a fluid stool from a patient be examined fresh, the amœba is seen to measure from 20 μ to 30 μ in diameter. It is of a faint green colour and is easily found owing to its ectoplasmic streaming.

and various
The outline
to the rapid
emergence of blunt pseudopodia in which the distinction between ectoplasm and endoplasm is easily seen. The amœba moves actively by a flowing motion. Its minute structures may better be observed in preparations which have been fixed while still wet in alcoholic corrosive sublimate solution and stained with iron-haematoxylin. The nucleus is found to have a definite, sharp, circular or oval outline, a central karyosome and a small amount of chromatin. The contractile vacuole of its con-
... may be
seen only rarely or not at all, since the entamœbæ have become encysted. The cyst, which is much smaller than the vegetative form, measuring from 10 μ to 15 μ in diameter, is round, and has a definite outline with double contoured wall. In its interior neither

life cycle in Man and the Animals:- (i) vegetative or trophic
(2) Precystic, (3) Cystic, (4) Metacystic stages.

It is well known that in malaria the patient may have remissions, during which no attacks of fever are experienced; but after a longer or shorter time typical bouts of fever recur, often as a result of a chill or some indiscretion. The explanation is probably that a certain degree of immunity is developed, and that the parasites live and go through schizogony in small numbers, possibly in some internal organ. It is only when a large number of red cells are attacked that fever develops. Very little real immunity is established against the parasite, but there may be some immunity against the toxic substances produced. Certainly negroes are less affected by malaria than are Europeans.

Quinine has for long been regarded as the most effective agent in the treatment of malaria, and its free administration appears to destroy the great majority of the asexual parasites. It is, however, without effect on the gametocytes. Among newer drugs, mepacrine (atebrin) and pamaquin (plasmoquin) have established themselves as of great value both in the treatment of malaria and in suppressing attacks of the disease. Paludrin appears to be a very promising drug for the treatment of the disease.

In the prophylaxis of malaria there are three cardinal factors: (1) Malaria is spread by the bites of mosquitoes; (2) mosquitoes bite at night; (3) mosquito bites are only dangerous if the insect has previously fed from an infected human being. The first necessity is to set about eradicating the mosquito. Since water is necessary for the development of the larva, all stagnant water should be drained or, where this is impossible, covered with oil, which prevents the development of the larva. While mosquitoes still exist, D.D.T. or other insecticide should be used and all those already infected should be treated in order to keep the number of parasites in their blood as low as possible. Europeans should sleep some distance away from infected natives, for the mosquito does not fly far. At night, the house or bed should be rendered mosquito-proof by wire or other netting. There is some discussion as to the value of a daily small dose of quinine in preventing the onset of the disease, and mepacrine is now commonly used as a prophylactic.

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a patient be examined fresh, the amœba is seen to measure from 20 μ to 30 μ in diameter. It is of a faint green colour, and can be easily found owing to its high refractivity. The outer zone, the

placed eccentrically, and also one or more vacuoles and various food substances, chiefly red blood cells and bacteria. The outline of the organism is constantly changing, owing to the rapid emergence of blunt pseudopodia in which the distinction between ectoplasm and endoplasm is easily seen. The amœba moves actively by a flowing motion. Its minute structures may better be observed in preparations which have been fixed while still wet in alcoholic corrosive sublimate solution and stained with iron-hæmatoxylin. The nucleus is found to have a definite, sharp, circular or oval outline, a central karyosome and a small amount of chromatin arranged in dots on the inner surface of its membrane. In the later stage of the disease, and during convalescence, the form of the *E. histolytica* described above may be seen only rarely or not at all, since the entamœbæ have become encysted. The cyst, which is much smaller than the vegetative

Life cycle in man and other mammals - 1. ... 2. ... 3. ... 4. ... 5. ... 6. ... 7. ... 8. ... 9. ... 10. ... 11. ... 12. ... 13. ... 14. ... 15. ... 16. ... 17. ... 18. ... 19. ... 20. ... 21. ... 22. ... 23. ... 24. ... 25. ... 26. ... 27. ... 28. ... 29. ... 30. ... 31. ... 32. ... 33. ... 34. ... 35. ... 36. ... 37. ... 38. ... 39. ... 40. ... 41. ... 42. ... 43. ... 44. ... 45. ... 46. ... 47. ... 48. ... 49. ... 50. ... 51. ... 52. ... 53. ... 54. ... 55. ... 56. ... 57. ... 58. ... 59. ... 60. ... 61. ... 62. ... 63. ... 64. ... 65. ... 66. ... 67. ... 68. ... 69. ... 70. ... 71. ... 72. ... 73. ... 74. ... 75. ... 76. ... 77. ... 78. ... 79. ... 80. ... 81. ... 82. ... 83. ... 84. ... 85. ... 86. ... 87. ... 88. ... 89. ... 90. ... 91. ... 92. ... 93. ... 94. ... 95. ... 96. ... 97. ... 98. ... 99. ... 100. ... 101. ... 102. ... 103. ... 104. ... 105. ... 106. ... 107. ... 108. ... 109. ... 110. ... 111. ... 112. ... 113. ... 114. ... 115. ... 116. ... 117. ... 118. ... 119. ... 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blood cells nor vacuoles are seen, but from one to four rounded nuclei with chromatin masses in

great ease, but the addition of a small amount of Gram's iodine facilitates their detection. In many cysts, probably those not yet mature, one or more elongated masses, the chromatoid bodies may be seen.

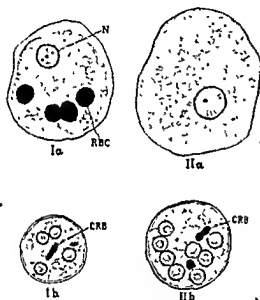


FIG. 97—ENTAMOEBAE.

Ia *E. histolytica*; N, nucleus; RBC, red blood cells. Ib *E. histolytica*, cyst; CRB, chromatoid body. IIa. *E. coli*. IIb *E. coli*, cyst.

As the diagnosis of the disease is made by the identification of the parasite in the stools, it is of great importance to distinguish it from another very similar organism, the ✓ *Entamoeba coli*, which is frequently found and which is non-pathogenic. This is usually somewhat larger than *E. histolytica*. In it the distinction between ectoplasm and endoplasm is not definite. ✓ the nucleus is much more easily seen, has an eccentric karyosome and coarse peripheral chromatin, and is situated near the centre,

The cysts are a resting stage in which the protozoa, in a resistant form, may have a better chance of reaching their natural habitat, the intestinal tract of a fresh victim. For this reason cysts are produced chiefly towards the end of the disease, at which period very few amœbæ may be found in the

stools. ✓ When swallowed, the amœbæ are destroyed by the gastric juice, but the cysts escape and reach the intestine. There they may settle down and multiply and act merely as saprophytes (*E. coli*), or either as saprophytes or pathogens (*E. histolytica*), for the latter organism does not in every case produce dysentery. In an experiment 20 men were given *E. histolytica* cysts by the mouth. In 18 the amœbæ were found in the stools, but only in four were the symptoms of dysentery noted. ✓ The possibility of this organism existing in the intestine for long periods without dysentery resulting explains the finding of it in stools of individuals who have never had dysentery, and is also probably of importance in the spread of the disease, since carriers may be either healthy or convalescent.

✓ In the disease in man the organism makes its way between the epithelial cells and so into the sub-mucosa of the large intestine, especially in the region of the cæcum and at the flexures of the colon. ✓ A gelatinous œdema results, with little leucocytic invasion of the part. ✓ Necrosis occurs, the slough separates off and an ulcer is produced. ✓ This may be from microscopic dimensions up to 3 cm. in diameter. ✓ It has irregular, overhanging edges and a ragged floor composed of the muscle coat. ✓ Amœbæ are found in the ulcer, particularly in the edges beyond the ulcerated area. ✓ The parasite may be carried in the blood or lymph to other parts of the body, most commonly the liver, where it frequently produces the so-called tropical abscess, of which one or more may be present. These are of irregular outline with ragged edges, and the chocolate or pinkish and blood with found in material they are entirely of

the vegetative form; cysts are not present.

The diagnosis of amœbic dysentery is made by finding either the characteristic amœbæ or cysts in the stools. ✓ The stool should be examined as fresh as possible in the wet state, films being made from a portion of blood-stained mucus when available, both with and without the addition of Gram's iodine.

✓ The disease is spread by the faeces of a patient or carrier, and infection may occur from water or food, particularly uncooked vegetables. Flies are probably important mechanical agents in transmitting amœbæ.

Dysentery may be produced in young cats and dogs by feeding with material containing the cysts of the *E. histolytica* or, more certainly, by the injection of such material into the rectum.

Cultures of some of the non-pathogenic entamœbæ have been made on a variety of media, but it has been found more difficult to cultivate *E. histolytica*. This has been accomplished by Boeck and Drbohlav using a medium consisting of solidified egg covered with dilute human serum. The cultivated entamœbæ exhibit characteristic pathogenicity for kittens.

Emetine is extensively used for the treatment of the disease with, in the majority of cases, successful results, and good reports have been received as to the value of chiniofon.

Trypanosomes

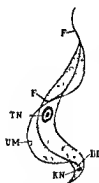


FIG. 98 — DIAGRAM OF TRYPANOSOME
F, Flagellum TN, Trophonucleus
UM, Undulating membrane BL, Blepharoplast.
KN, Kinetonucleus

The trypanosomes are motile protozoa, living in the blood plasma of man or of the lower animals, and producing in these a variety of diseases. Minor differences characterise each species, but fundamentally they are very similar. A trypanosome has an elongated body, measuring from 15μ to 30μ in length by 1.5μ to 2.5μ in breadth in the thickest part, from which it tapers towards each extremity. At one pole, the anterior, is a long, fine flagellum which commences near the posterior end in the blepharoplast, close to the kinetonucleus, and is continued anteriorly along the edge of a delicate, indented, fin-like structure, the undulating membrane. In its interior, near the centre in the stained specimen, is seen a round or oval nucleus, while near the posterior pole is a much smaller structure, the kinetonucleus. The organism swims

actively through the plasma, disturbing the blood cells as it goes. Its motility appears to be due to the flagellum and to contractions of its body, but chiefly to the undulating membrane. In the vertebrate body multiplication takes place by longitudinal division. The two resulting trypanosomes may be equal in size, but commonly one is very much smaller than the other. In the same blood considerable variations in the parasites may be found, and these have been regarded by some as male and female forms. Sexual reproduction has not, however, been observed in trypanosomes. The life-cycle of these flagellates involves an alternation of hosts,



FIG 99—*Trypanosoma evansi* IN RAT BLOOD (X 950)

one a vertebrate, the other a blood-sucking insect, in which the most essential part of the developmental process takes place.

In man the chief disease due to trypanosomes is sleeping sickness, one of the most serious of the diseases of tropical Africa, which is caused by *T. gambiense*. The disease commences with irregular fever, wasting, muscular weakness, enlargement of the spleen, and the occurrence of areas of local erythema and œdema. The condition is rather chronic, but may cause death. If death does not occur at this stage, after a longer or shorter time, a change in disposition is observed. The patient becomes morose and apathetic; the speech is slow and headache and indefinite

pains are complained of; the superficial lymphatic glands are enlarged; muscular tremors occur, and there is progressive emaciation and anæmia; the lethargy becomes more marked and passes into coma, followed by death. The total duration of the disease may be several years. The chief pathological changes are congestion and inflammation of the meninges and, in the brain, a perivascular infiltration with lymphocytes.

In the early stages of the disease the trypanosomes are found in the blood, but not in large numbers. Later they are present in the cerebro-spinal fluid and in the juice of the enlarged glands.

Injection of material containing the parasites into monkeys gives rise to a disease closely resembling that of man. Dogs, cats, and white mice are also susceptible, and guinea-pigs to a much less extent.

The disease is spread by the bite of *Glossina palpalis* (the tsetse fly), of which both male and female bite in daylight. This insect may transmit the disease mechanically for a short time after the infecting feed. Then, for a period of several weeks, a bite is without effect, but subsequently to this, biting may reproduce the disease and the insect may remain infective for a considerable time. The trypanosome goes through a complicated cycle of development in the tsetse which ends in the presence of infective trypanosomes in the salivary glands of the insect. *Tr. gambiense* can live in the blood of wild animals, and it appears probable that the disease is kept alive in a district by antelopes and other big game acting as reservoirs of the organism.

Animals may, to some extent, be immunised against this trypanosome and their sera are found to destroy the organism and to protect other animals against infection. Serum useful for the treatment of the disease in man has, however, not yet been produced.

Sleeping sickness, when fully developed, is always fatal if untreated, but the earlier manifestations may become arrested and the lethargic stage may not be reached. Many drugs have been tried, of which the most promising until recently were certain arsenical derivatives (atoxyl, salvarsan, etc.) and salts of antimony.

These undoubtedly are very effective in reducing the number of parasites in the blood, and can produce cures in experimentally infected animals. A certain number of human infections appear to have been definitely cured, but success has not usually attended the use of these drugs once the central nervous system has been attacked. A few apparent cures have, however, been reported following the use of tryparsamide in cases in which the central nervous system was involved. Suramin and antrycide have been used with apparently good results in a large number of cases, especially in the early stages of infection with *Tr. gambiense*.

One great difficulty and danger in the treatment of diseases due to trypanosomes is that, if the patient receives inadequate doses of a drug, his trypanosomes may become drug-resistant so that they are unaffected by a concentration of the drug lethal to normal trypanosomes. This resistance is usually called arsenic-fastness, since it was first observed with arsenical preparations. It is not, however, a resistance to arsenic, but to the substituted phenyl radicals of the aromatic compounds used. Resistance is maintained for many generations of the trypanosome and has been found still present after 5 years, during which the organisms were passaged through hundreds of mice and tsetse flies. There are reasons for believing that, in parts of Africa, resistant strains are becoming increasingly common as the cause of human disease. It is, therefore, important in the interests both of the patient and of the community, that dosage should be adequate, especially in the case of aromatic arsenical and antimonial compounds.

Tr. rhodesiense is an organism almost identical with the *Tr. gambiense*, but causing in man a more rapid and virulent form of the disease. As a result of immunisation experiments in animals it is found to be a distinct organism serologically. Many experienced workers believe that *Tr. rhodesiense* is the same organism as *Tr. brucei*, the cause of nagana in horses, possibly modified by passage through man. It certainly is transmitted by the same fly, *Glossina morsitans*.

A considerable number of trypanosomes are found as parasites in other animals. The most widespread of these is *Tr. lewisi*,

found in the blood of rats in various parts of the world. The animal seems to be little injured by the presence of the organism and to acquire immunity to it after a few months, when the blood is found to be free of trypanosomes. The parasite is transmitted by fleas in which the infective trypanosomes are produced in the hind gut and voided with the faeces, infection taking place through faecal contamination.

Nagana, a very fatal disease affecting animals in various parts of Africa, is due to *Tr. brucei* (horses and dogs), *Tr. congolense* or *Tr. vivax* (cattle). The disease is conveyed by a tsetse fly, *Glossina morsitans*, and the reservoirs from which the flies are infected are the antelopes and other wild animals of the part, which are apparently unaffected by the presence of the parasites. *Tr. evansi* also affects horses, producing in India the disease known as surra.

Tr. cruzi, a trypanosome which differs considerably in morphology from those described above, is the cause of Chagas' disease, which occurs in Brazil and other parts of South America. The parasite is transmitted by a bug, *Triatoma megista*.

Diagnosis of African trypanosomiasis is usually made by the finding of the trypanosome in blood, cerebro-spinal fluid, or in gland juice. In the case of blood infections, the fluid should be diluted with saline and examined as a wet preparation. Films should also be prepared and stained by Leishman's stain. If the number of parasites is small, the centrifuging of citrated blood and examination of the upper layer of deposited cells may facilitate the search. Cerebro-spinal fluid usually requires prolonged centrifuging. Inoculation of animals—preferably monkeys, dogs or rats—with suspected material and the subsequent finding of the parasite in the blood may occasionally enable a diagnosis to be made.

The control of the diseases caused by trypanosomes requires the extinction or at least the reduction of the transmitting flies by the use of the newer insecticides. It has been found that 1.0 to 1.5 g. of suramin, injected intravenously, renders a man insusceptible to infection by trypanosomes for more than 3 months.

Leishmaniasis

Kala azar, known also as Dum-dum fever and by many other names, is a fairly common disease occurring in India, China, Malay and in many other tropical countries, either sporadically or, more rarely, in epidemic form. It is a chronic disease in which the rate of mortality in untreated cases is very high. Its outstanding features are irregular fever, emaciation, anæmia, enlargement of the spleen and liver, local œdema, and the occurrence of ulcers both of the skin and intestine, especially the colon.



FIG 100.—*Leishmania donovani* IN SECTION OF SPLEEN
 V S Venous sinus L C Lymphoid cell R Reticulum E Large endothelial
 phagocytic cell L *L. donovani* A *L. donovani* (greatly enlarged) B
Leptomonas form of *L. donovani* (greatly enlarged)

The causal organism (*Leishmania donovani* or the Leishman-Donovan body) is found in enormous numbers in films made from the spleen or liver. It is most usually oval or of cockle-shell shape, from 2μ to 4μ in greatest diameter. When stained with Leishman's stain it is of a faint blue colour. In the interior are to be seen two bodies.—the oval or irregular nucleus, which stains pink, and a smaller, a rod-shaped body, the kinetoplast, which stains a dark red colour. One or more vacuoles may also

be distinguished. The parasites are most usually seen in the interiors of large mononuclear (endothelial) cells. They multiply by simple fission until the host cell is distended and bursts, liberating them, when they invade new cells. The Leishman-Donovan bodies are most frequently seen in the spleen, liver and bone marrow and, in smaller numbers, in the lung, kidney, lymphatic glands and ulcers of the skin and intestine, almost always in the endothelial cells of blood vessels and lymphatics. They may also be found, after prolonged search in many cases, in the peripheral blood, usually in either mononuclear or polymorphonuclear leucocytes.

The organism can be cultivated in the medium of Novy and MacNeill used for trypanosome culture. In this medium the organism enlarges, becomes elongated and develops a flagellum (leptomonas form). It then bears some resemblance to a trypanosome, but no undulating membrane is present.

The organism is pathogenic for monkeys, rats and mice and, to a less extent, for dogs, guinea-pigs and rabbits. Infection is most certainly produced by intraperitoneal or intrahepatic injection of large amounts of an emulsion of infected tissue or of culture. In a few experiments, monkeys have been infected by feeding. The occurrence of the parasite in the skin ulcers, in discharges from the mouth and nose and in the faeces, renders direct or indirect infection possible. Dogs may act as reservoirs of the organism; but naturally infected dogs are not commonly found in India and artificial inoculations frequently fail to produce the disease in them. The invertebrate host is a sandfly (*Phlebotomus*) which transmits the infection by its bite.

About the shores of the Mediterranean a very similar disease is found, except that it is confined almost entirely to children aged from 2 to 5 years. An organism (*Leishmania infantum*); which is identical morphologically with *L. donovani*, is found in the same situations in the body as is the latter parasite in kala azar. Many believe that *L. infantum* is really *L. donovani*, the virulence of which has been modified by passage. Where this disease occurs, many dogs are found to be naturally infected. It is probable that

in the case of this disease the dog acts as a reservoir and that the organism is transmitted by the sandfly, dog flea or other insect.

Diagnosis of the diseases may be made by spleen puncture which, if proper precautions are taken, is not dangerous in kala azar: liver puncture often reveals the presence of the parasites. Prolonged search of blood films may also show the Leishman-Donovan bodies, or these may be found in cultures of the blood. If enlarged lymphatic glands are present, some of the juice may be obtained by gland puncture and examined. The leucopenia, chiefly

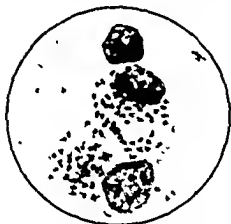


FIG 101 —*Leishmania tropica* IN SMEAR
FROM ORIENTAL SORE ($\times 950$)

affecting the polymorphonuclear leucocytes, may assist diagnosis.

Another condition (Oriental sore, Bagdad button, or Delhi boil) is due to a similar organism. On an exposed part of the body a papule develops. This enlarges, becomes covered with scales and breaks down, leaving an ulcer. The ulcer enlarges and is unaffected by external treatment, healing only occurring after many months.

The organism producing the condition, *L. tropica*, is in almost all respects identical with *L. donovani*. It is found in the discharge or at the edges of the ulcer, usually within endothelial cells.

The disease is inoculable and is transmissible to dogs and monkeys, either by injection of the discharge or of cultures. Infection may be direct or indirect through the agency of flies or other insects. *L. tropica*, like *L. donovani* is transmitted by sandflies.

It is usually found that one attack confers a lasting immunity, and in animals experiments have shown a certain degree of immunity against *L. donovani* as a result of infection with *L. tropica* and vice versa. The treatment of visceral Leishmaniasis has been greatly improved since the introduction of various pentavalent organic antimony compounds and more recently of the diamidines, of which the most satisfactory is pentamidine.

Ref: ① Hand Book of Medical Protozoology By C.A.H.
 (London) B.T.C. 1949 1st edition.

② An Introduction to Medical Protozoology.
 By Robert Knowles. (Calcutta) The Press,
 Spink & Co Ltd. 1922. Calcutta.

CHAPTER XLVII

PATHOGENIC FUNGI

Certain pathological conditions either of the skin or, less commonly, of the subcutaneous tissues or of the viscera, have been found to be due to fungi. The most important of these resemble in many ways the moulds, but some are similar to the yeasts. In all these organisms we find that the morphology and methods of reproduction are of the simplest character while they are parasites, but when growing saprophytically, as on artificial media, the structure and mode of reproduction of many of them are complicated, and enable them to be grouped with the moulds commonly found in damp places under natural conditions.

Parasitic mycology is now a subject of considerable magnitude, and here it is impossible to give more than a brief outline of some of its most important points. A word as to the special methods involved is first necessary. In ringworm of the scalp the hairs to be examined should be taken from around the edge of the affected area. A convenient method of obtaining material from the nails is to scrape them with a microscope slide which has been broken, leaving a sharp edge. Epithelial scales from the skin may be detached with a forceps. For microscopic examination of the parasites in hairs and epithelial scales, the simplest method is to place the fragment in a drop of 40 per cent potassium hydrate solution on a slide and cover with a cover slip. The slide is then gently warmed, and in a few moments hairs are ready for examination. A much longer time (2 or 3 hours) is advisable in the case of epithelial scales. The alkali clears the tissue and the details of the fungus can be made out quite well. Staining methods are troublesome and not always satisfactory.

Many of the parasites can be cultured, and a suitable medium is agar containing 1 per cent. of peptone without meat extract,

but with 4 per cent. of either maltose or glucose. The reaction should incline to the acid rather than to the alkaline side of neutrality. Aerobic conditions are essential and a temperature somewhat below that of the body usually gives best results, although growth takes place either at air or at body temperature. One noteworthy feature of the cultures of these organisms is that very slight differences in the composition of the medium, even such as that found in two batches of media made according to the same formula, exercise great differences in the type of colony produced. For this reason it is difficult in many cases to recognise a fungus from the appearance or colour of the colony.

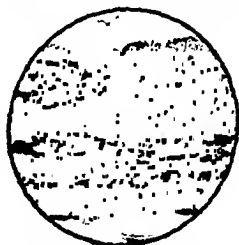


FIG 102.—*Microsporon audouinii* IN HAIR
($\times 250$)

Ringworm is of two chief types, and these are due to the action of two different varieties of fungi. The ringworm which occurs almost exclusively in children, particularly on the scalp, is due to a *Microsporon*; that found in adults on the beard, hairless skin and nails and very rarely on the scalp, is caused by a *Trichophyton*.

Microsporon audouinii is found in affected hairs and epithelial scales as filaments, which have a diameter of about 2μ to 5μ . These first appear in the superficial part of the epidermis, but soon they penetrate the hairs at the level at which they emerge from their sheaths. The filaments, which grow along the hair down-

wards and for a short distance upwards, when carefully examined, are found to be branched. The filaments are not continuous, but their protoplasm is separated by cross divisions, which occur at some distance from one another. In many cases the hairs may show but slight signs of invasion, since the most marked activity of the parasite is not in the interior but on the exterior of the infected hair which it covers. Towards the ends of branches the septa occur at closer and closer intervals, so that these branches end in a short chain of small, irregularly spherical spores measuring from 2μ to 3μ in diameter. The whole of the exterior of the hair is covered with a layer composed of enormous numbers of these spores so tightly pressed together that they have assumed a polyhedral shape.

This parasite affects man exclusively, and occurs but rarely after the age of puberty. It has not commonly been found possible to infect either rabbits or guinea-pigs, and only a few successful inoculations have been recorded. There are, however, other varieties of microspora, which occur naturally in animals but are also pathogenic for man.



FIG. 103.—COLONY OF *Microsporon audouinii* ON GLUCOSE AGAR ($\times 1$)

On glucose or maltose agar *M. audouinii* grows well. The colonies, after about a fortnight, are large, of a white colour and covered with a delicate downy layer. In the centre there is usually a distinct elevation from which furrows pass towards the periphery, or occasionally an arrangement of concentric rings is seen. In cultures other methods of reproduction are observed—chlamydospores which are swollen, ovoid cells situated in the course of certain hyphæ, and conidia, small spores 2μ to 3μ by 3μ to 4μ arranged along the sides of hyphæ. Cultures of animal microspora may be distinguished from those of *M. audouinii* by the occurrence of large spindle-shaped bodies measuring from 15μ to 20μ by 40μ to 60μ . These contain a number of cells and are situated at the free ends of hyphæ.

Ringworm caused by various species of *Trichophyton* affects

but with 4 per cent. of either maltose or glucose. The reaction should incline to the acid rather than to the alkaline side of neutrality. Aerobic conditions are essential and a temperature somewhat below that of the body usually gives best results, although growth takes place either at air or at body temperature. One noteworthy feature of the cultures of these organisms is that very slight differences in the composition of the medium, even such as that found in two batches of media made according to the same formula, exercise great differences in the type of colony produced. For this reason it is difficult in many cases to recognise a fungus from the appearance or colour of the colony.

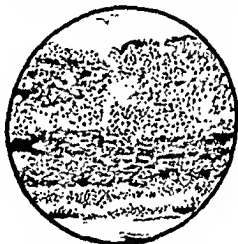


FIG 102 — *Microsporon audouinii* IN HAIR
($\times 250$)

Ringworm is of two chief types, and these are due to the action of two different varieties of fungi. The ringworm which occurs almost exclusively in children, particularly on the scalp, is due to a *Microsporon*; that found in adults on the beard, hairless skin and nails and very rarely on the scalp, is caused by a *Trichophyton*.

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occur in man and animals, both in the interior and on the exterior of the hair. In a hair infected with a human *Trichophyton*, branched mycelial filaments almost fill the hair, but in these the septa are so closely arranged that only chains of spores, which measure from 4μ to 6μ in diameter, are seen. In the animal trichophyta the filaments are found both inside and around the hair. The spores in this case are more irregularly arranged

Different types of both human (*T. crateriforme*, *T. acuminatum*) and animal (*T. gypseum*, *T. felinum*, etc.) trichophyta may be distinguished by their cultural characteristics. The colonies are usually white or grey, but in some a brown or violet colour may develop later. The central portion is elevated, with or without a small depression. Around the periphery delicate branches spread over the surface of the medium. The whole is covered with a fine downy layer. Some types show a marked arrangement of furrows running radially from the centre towards the periphery. In cultures, the reproductive organs include conidia, which differ from those of *Microsporon*, since the spores are produced at the ends of short branches arranged along the course of a hypha, while in the *Microsporon* the conidia are attached directly to the hypha. Large spindle-shaped organs occur which resemble those of a *Microsporon*, but the chlamydospores are round and not ovoid. In the trichophyta the terminal portion of certain hyphae is found to be arranged as a close spiral with from 8 to 10 turns. The object of these spiral filaments is unknown, but their occurrence is sufficient to distinguish a *Trichophyton* from a *Microsporon*.



FIG. 106 — COLONIES OF *Trichophyton* ON GLUCOSE AGAR ($\times 1$)



FIG. 107 — COLONIES OF *Achiorion schenleini* ON GLUCOSE AGAR ($\times 1$)

man at all ages. These fungi may cause disease in the beard, smooth parts of the body or in the scalp and nails. In these forms of ringworm there is more inflammation, and pus may be present.



FIG. 104.—*Trichophyton endothrix* IN HAIR ($\times 250$).

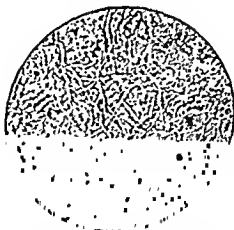


FIG. 105.—*Trichophyton megalosporon* IN SCALE OF SKIN ($\times 250$)

The parasites may be divided into two types—the endothrix, confined to the human race, in which the organism is found chiefly in the interior of the hairs; and the endo-ectothrix types, which

from an unopened abscess should be obtained and thickly spread on glucose or maltose agar slopes. The colonies, which at first are light in colour, darken to a chocolate brown as they get older. The surface is convoluted, and frequently the centre shows a depression.



FIG. 103.—COLONIES OF *Sporotrichum beumanni* ON GLUCOSE AGAR ($\times 1$)

Around the periphery of the colony a white border is seen.



FIG. 109.—*Monilia* FROM THROAT CULTURE ($\times 700$).

The serum of a patient suffering from sporotrichosis agglutinates the spores of the fungus in many cases up to 1 : 1000. The presence of other fungi or of actinomyces in the body may cause a false agglutination, and for diagnosis a titre of not less than 1 : 200 is essential. The sporotricha are pathogenic for other animals—the mouse, rat and dog especially—and in them produce granulomata or multiple abscesses in the internal organs. Potassium iodide is an almost specific treatment for the condition.

Thrush is a disease chiefly of childhood, in which white patches occur on the mucous membrane of the mouth or pharynx. The

Favus, which affects chiefly the scalp of children, is almost always due to the *Achorion schaeleini*. A cup-shaped crust, the scutulum, which is of a yellow colour, forms about a hair. The scutulum is made up of a felted mass of fine, septate, branching filaments, measuring from 2μ to 3μ in diameter. In the central part the cross divisions approximate, leading to the production of rectangular spores. In the hair itself chiefly the spores are seen, and these show considerable variations in size.

The colonies are of a grey or brown colour. They may be cup-shaped, but more usually show a warty or convoluted pattern. The edge is generally sharply defined and without the finely branching arrangement seen frequently in the microspora and trichophyta. The surface may appear to be covered with fine powder, but is not downy like the colonies of the ringworm organisms. In cultures, one of the most characteristic features is the occurrence of ovoid bodies of a yellow colour, situated usually at the ends of the filaments and measuring about 10μ in diameter. Conidia are produced on the ends, or along the sides of some filaments. *Achorion schaeleini* is only slightly pathogenic for animals. Of these the mouse is most easily infected, and in that animal a scutulum is produced. Other varieties, of which the most important is *Achorion quinckeum*, occur naturally on other animals and are capable of infecting man.

Sporotrichosis is a rather rare condition in which granulomata are found, most commonly in the subcutaneous tissue and less often in the bones and viscera. The granulomata, which at first are hard and elastic, soften and break down, erupting on the surface through fistulae from which serous pus is discharged. The lymphatic vessels become hard and thickened and along their course nodules may develop which also ulcerate. The lesions, which are very chronic, are commonly mistaken for those of syphilis or tuberculosis. In the discharge the causative organism, *Sporotrichum beurmanni* or *S. schenki* may be found either in the form of oval spores 3μ to 6μ in length or, less commonly, as a mycelium. In the majority of cases, however, no parasite is discovered microscopically in the discharge. For culture, the pus

from an unopened abscess should be obtained and thickly spread on glucose or maltose agar slopes. The colonies, which at first are light in colour, darken to a chocolate brown as they get older. The surface is convoluted, and frequently the centre shows a depression.



FIG 108—COLONIES OF *Sporotrichum beurmanni* ON GLUCOSE AGAR ($\times 1$)

Around the periphery of the colony a white border is seen.



FIG 109—*Monilia* FROM BROTH CULTURE ($\times 700$)

The serum of a patient suffering from sporotrichosis agglutinates the spores of the fungus in many cases up to 1 : 1000. The presence of other fungi or of actinomyces in the body may cause a false agglutination, and for diagnosis a titre of not less than 1 : 200 is essential. The sporotricha are pathogenic for other animals—the mouse, rat and dog especially—and in them produce granulomata or multiple abscesses in the internal organs. Potassium iodide is an almost specific treatment for the condition.

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EHRlich'S REAGENT

<i>Para</i> -dimethylamidobenzaldehyde	1 g.
Absolute alcohol	95 ml.
Concentrated hydrochloric acid.	20 ml.

GLUCOSE

Glucose	100 g.
Water	1000 ml.

Dispense in 10 ml. amounts in tubes or bottles and sterilise by autoclaving at 115° for 15 minutes.

SALINE

Sodium chloride	8.5 g.
Water	1000 ml.

Dispense in tubes, bottles or flasks and sterilise by autoclaving at 115° for 15 minutes.

STAINING SOLUTIONS

In the case of staining solutions containing alcohol and water, the dry stain should be dissolved in the alcohol and the water added to the solution.

All staining solutions should be filtered a few days after they have been prepared.

Some staining solutions (*e.g.* carbol fuchsin) are liable to precipitation. They should be filtered directly on to the slide.

ACID ALCOHOL

Hydrochloric acid	3 ml.
95 per cent. alcohol	100 ml.

ALBERT'S STAIN

Toluidin blue	0.15 g.
Malachite green	0.20 g.
95 per cent. alcohol	2 ml.
Glacial acetic acid	1 ml.
Water	100 ml.

ALKALINE METHYLENE BLUE (LÖFFLER'S) STAIN

Saturated alcoholic solution of	
methylene blue	30 ml.
1 per cent. aqueous potassium	
hydrate solution	1 ml.
Water	100 ml.

Polychrome methylene blue is better for the staining of *C. diphtheriæ*. This is alkaline methylene blue which has been ripened by storage for several months in half-filled bottles which are shaken at intervals.

BISMARCK BROWN STAIN

Bismarck brown	1 g.
Water	500 ml.

CARBOL FUCHSIN STAIN

Basic fuchsin	1 g.
95 per cent. alcohol	10 ml.
Phenol	5 g.
Water	100 ml.

DILUTE CARBOL FUCHSIN STAIN

Carbol fuchsin stain	1 part
Water	14 parts

CARBOL THIONIN BLUE STAIN

Thionin	1 g.
Phenol	2.5 g.
Water	100 ml.

For use, dilute 1 in 4 with water.

CHRYSOIDIN STAIN

Chrysoidin	1 g.
Water	300 ml.

CRYSTAL VIOLET STAIN

Crystal violet	0.5 g.
Water	100 ml.

FONTANA'S STAIN

1. Acid Formalin Solution (fixative)

Acetic acid	1 ml.
Formalin	2 ml.
Water	100 ml.

2. Tannic Acid Solution (mordant)

Tannic acid	5 g.
Phenol	1 g.
Water	100 ml.

3. Silver Solution

1 per cent. aqueous silver nitrate solution 5 ml.

To this add, drop by drop, 10 per cent. aqueous solution of ammonium hydrate. Continue the addition with constant shaking until the turbidity, which appears, almost completely disappears. If the fluid becomes completely clear, add more of the silver nitrate solution until faint turbidity appears and persists.

N.B.—This solution should not be kept for more than a few hours as, on keeping, a violent explosive forms.

LEISHMAN'S STAIN

Leishman's powder	0.15 g.
Methyl alcohol (pure, acetone free)	100 ml.

LUGOL'S IODINE

Iodine	1.0 g.
Potassium iodide	2.0 g.
Water	100 ml.

MALACHITE GREEN STAIN

Malachite green	0.5 g.
Water	100 ml.

METHYL VIOLET STAIN

Methyl violet (6B)	0.5 g.
Water	100 ml.

NEISSER'S METHYLENE BLUE STAIN

Methylene blue	1 g.
95 per cent. alcohol	50 ml.
Glacial acetic acid	50 ml.
Water	950 ml.

NEUTRAL RED STAIN

Neutral red	1.0 g.
1 per cent. aqueous acetic acid solution	20 ml.
Water	1000 ml.

NEUTRAL RED AND CARBOL FUCHSIN STAIN

Carbol fuchsin stain	50 ml.
Neutral red stain	950 ml.

NIGROSIN SOLUTION

Nigrosin (Gurr)	10.0 g.
Water	100.0 ml.
Formalin	0.5 ml.

PAPPENHEIM'S STAIN

Methyl green (free from methyl violet)	1.0 g.
Pyronin	0.25 g.
95 per cent. alcohol	50 ml.
Glycerol	200 ml.
Phenol	20 g.
Water	100 ml.

PUGH'S STAIN

Toluidin blue	0.1 g.
95 per cent. alcohol	50 ml.
Glacial acetic acid	20 ml.
Water	100 ml.

SAFRANIN STAIN

Safranin	10 g.
Water	100 ml.

CULTURE MEDIA

Media for General Purposes

BROTH (WRIGHT'S METHOD)

Peptone	10 g.
Sodium chloride	5 g.
Minced meat, free from fat	500 g.
Water	1000 ml.

Mix. Heat at 68° for 20 minutes with constant stirring. Steam 30 minutes. Filter through paper. Adjust to pH 8.0. Steam 30 minutes. Filter through paper. Adjust, if necessary, to pH 7.4 with dilute HCl. Dispense in tubes or bottles. Sterilise in the autoclave at 115° for 15 minutes.

LEMCO BROTH

Lab. Lemco	5 g.
Peptone	10 g.
Sodium chloride	5 g.
Water	1000 ml.

Steam 30 minutes. Adjust to pH 8.0. Steam 30 minutes. Adjust to pH 7.4. Filter through paper. Dispense in tubes or bottles. Sterilise in the autoclave at 115° for 15 minutes.

PEPTONE WATER

Peptone	10 g.
Sodium chloride	5 g
Water	1000 ml.

Steam Adjust to pH 7.4. Filter. Dispense in tubes or bottles
Sterilise in the autoclave at 115° for 15 minutes.

AGAR

Agar powder	20 g
Broth	1000 ml.

Steam 1 hour. Adjust to pH 7.4. Steam 1 hour. Filter (in steamer or jacketed funnel) through paper or cotton wool. If clear medium is required, add the white of two eggs beaten up with a little water to the medium cooled to 50° before second steaming. Dispense in tubes or bottles. Sterilise in the autoclave at 115° for 15 minutes.

GELATIN

Gelatin	150 g.
Broth	1000 ml.

Steam 30 minutes. Adjust to pH 7.4. Add egg as for agar.
Steam $\frac{1}{2}$ hour. Filter. Dispense in tubes or 1 oz. round, screw cap bottles Sterilise in the steamer by the intermittent method.

SUGAR MEDIA

Carbohydrate	1 g.
Andrade's indicator	1 ml.
Peptone water (pH 7.2)	100 ml.

The medium, when cold, should be practically colourless. At a temperature of 100°, it should be slightly pink. The above holds good for the usual carbohydrates (lactose, glucose, maltose, saccharose and mannitol). Dulcitol is used in a concentration of 0.5 per cent Sugar media, dispensed in Durham tubes, are sterilised in the steamer by the intermittent method.

LACTOSE BILE SALT BROTH

Sodium taurocholate	5 g.
Peptone	20 g.
Water	1000 ml.

Dissolve by boiling. Adjust to pH 7.2. Stand overnight.

Filter. Add:

Lactose	10 g.
Andrade's indicator	10 ml.

Dispense in Durham tubes and bottles. Sterilise in steamer by intermittent method.

Media for the Culture of *C. DIPHTHERIÆ*

COAGULATED SERUM MEDIUM (LÖFFLER)

Serum	300 ml
Broth containing 1 per cent. glucose	100 ml.

Dispense in tubes. The amount added to each should be such that, when the tube is sloped at an angle of 15° with the horizontal, the fluid reaches from one-third to one-half way up the tube. They are so arranged in an inspissator, the temperature of which is slowly raised to 85° at which it is held for 2 hours. If the serum was sterilised by filtration through a Seitz filter, this will be sufficient to sterilise it. If not, the slopes should be sterilised in the upper part of a steamer by the intermittent method. Serum slopes should be incubated for 2 days prior to use to detect any which are contaminated. This medium deteriorates quite rapidly in tubes as a result of drying. Unless the turnover is considerable it is, therefore, advisable to use 1 oz. round, screw cap bottles in place of tubes. These, each containing 5 ml. of the mixture, should be inspissated in the horizontal position.

HOYLE'S MEDIUM

Citrated horse blood (sterile)	100 ml.
10 per cent. sterile saponin solution	5 ml.

(1)

Warm the blood to 37° in incubator, add the saponin solution, mix without frothing, incubate 15 minutes.

If precautions to maintain sterility are observed, the resulting solution can be kept in a screw cap bottle in the refrigerator for several months.

(2)

Potassium tellurite	3.5 g.
Water	100 ml

Keep in screw cap bottle in the dark

(3)

Melt 100 ml. agar made from Lemco broth (preferably with Difco proteose peptone), cool to 55°, add 5 ml blood preparation and 1 ml. tellurite solution. Mix. Pour plates

Media for the Isolation of Intestinal Bacteria

BUFFERED GLYCEROL SALINE

Sodium chloride	0.6 g.
Disodium hydrogen phosphate (anhydrous)	1 g.
Glycerol	30 ml.
0.02 per cent. aqueous phenol red	1.5 ml.
Water	70 ml.

Heat in steamer to dissolve

Dispense in wide mouth, screw cap bottles. Sterilise in autoclave at 110° for 15 minutes. This solution is used to preserve specimens of faeces for culture of intestinal pathogens. If the pink colour changes to yellow, indicating the development of an acid reaction, it is useless.

MACCONKEY'S MEDIUM

Sodium taurocholate	5 g.
Agar powder	20 g.
Peptone water	1000 ml.

Steam 1 hour. Adjust to pH 7.4. Steam 1 hour. Filter as for agar. Add

Lactose	10 g.
1 per cent. aqueous neutral red	2.5 ml.

Dispense in tubes and bottles. Sterilise in the steamer by the intermittent method.

HANDBOOK OF BACTERIOLOGY DESOXYCHOLATE-CITRATE MEDIUM (Leifson's Medium as modified by Hynes)

(1)

Lab. Lemco	20 g.
Peptone (Disco proteose)	20 g.
Water	200 ml.

Dissolve with aid of heat. Adjust pH to 9.0 with 50 per cent. NaOH solution. Boil. Filter. Adjust to pH 7.3. Make up volume to 200 ml.

(2)

Agar powder	90 g.
Water	3700 ml.

Dissolve in steamer. Filter.

(3)

Mix 1 and 2 while both are still hot. Add:

2 per cent. aqueous solution of neutral red	5 ml.
Lactose	40 g.

Bottle in accurate 100 ml. volumes. Sterilise in steamer by intermittent method.

(4)

Sodium citrate (crystals) (A.R.).	17 g.
Sodium thiosulphate (crystals) (A.R.)	17 g.
Ferric citrate (Scale)	2 g.
Water	100 ml.

Dissolve with gentle heat. Heat for 1 hour at 60° in screw cap bottle.

(5)

Sodium desoxycholate	10 g.
Water	100 ml.

Heat for 1 hour at 60° in screw cap bottle.

For use:

No. 3 (melted in steamer)	100 ml.
No. 4	5 ml.
No. 5	5 ml.

Use sterile precautions. Use separate pipettes for No. 4 and

No. 5 and mix well after each addition. Pour plates immediately and dry the surface in the incubator. The final medium does not keep. The three ingredients (Nos 3, 4 and 5) can be kept for months

WILSON AND BLAIR'S MEDIUM

(1)

Bismuth-ammonium-citrate (scale preparation)	30 g.
Water	250 ml.

Boil.

(2)

Sodium sulphite (anhydrous)	100 g.
Water	500 ml.

Boil

(3)

Mix 1 and 2 while both are at boiling point and at once add:
Sodium phosphate ($\text{Na}_2\text{HPO}_4, 12\text{H}_2\text{O}$) 100 g.

Cool.

(4)

Glucose	50 g.
Water	250 ml

Boil. Cool.

(5)

Mix 3 and 4.

(6)

Ferric citrate (scale preparation)	2 g.
Brilliant green	0.25 g.
Water	200 ml.

7

No. 5	20 ml
No. 6	4.5 ml
Nutrient agar	100 ml.

The agar is melted and cooled to 60° before the addition of Nos 5 and 6. These should be well mixed before the necessary

amounts are measured as any deposits which have formed must be included. The whole is mixed and poured into plates which are dried in the incubator before use. Nos. 5 and 6 keep for months. The final medium should be used on the day it is prepared.

TETRATHIONATE BROTH

(1)

Chalk	25 g.
Broth	900 ml.

The chalk should have been autoclaved and dried before being added to the broth.

Steam for 30 minutes.

(2)

Sodium thiosulphate (crystals) .	600 g.
Water to	1000 ml.

Steam for 30 minutes.

(3)

Iodine	60 g
Potassium iodide	50 g.
Water to	200 ml.

Grind the iodine and potassium iodide in a mortar before the water is added.

(4)

No. 1	18 ml
No. 2	20 ml.
No. 3	4 ml.

Mix the constituents before measuring and the mixture before dispensing. Dispense, with sterile precautions, in sterile tubes. 5 to 10 ml. in each.

The medium as prepared for use should not be kept for more than a few days. The constituents 1, 2 and 3 keep indefinitely.

SELENITE MEDIUM

Sodium acid selenite	4 g.
Peptone	5 g.
Lactose	4 g.
Disodium hydrogen phosphate (Na_2HPO_4)	9.5 g.
Sodium dihydrogen phosphate (NaH_2PO_4)	0.5 g.
Water	1000 ml.

The peptone should be dissolved in the water with the aid of heat and the other ingredients added when the peptone solution is cool. The pH of the medium should be adjusted to 7.1 by further addition of either Na_2HPO_4 or NaH_2PO_4 . Dispense in 10 ml. amounts in tubes or screw cap bottles. Sterilise by steaming for 30 minutes. The formation of a slight red precipitate is of no importance.

Media for the Culture of Tubercle Bacilli

GRIFFITH'S EGG MEDIUM

Eggs, not more than 24 hours old, are scrubbed, immersed in boiling water for 10 seconds and then in spirit. When dry, they are opened, with aseptic precautions, and the contents transferred to a sterile graduated cylinder. To the eggs one-half their volume of sterile saline is added. The mixture is shaken, with a rotary movement, in a sterile flask for 20 minutes and is filtered through sterile gauze into a sterile flask. A sufficient amount of a solution of basic fuchsin is added to give the medium a pale pink colour. The mixture is dispensed in 5 ml. amounts in 1 oz. screw cap bottles which are inspissated in a horizontal position at 85° for 45 minutes. Next day they are held at the same temperature for $1\frac{1}{2}$ hours.

Glycerol egg medium is prepared by adding to the egg-saline mixture sufficient glycerol to give a concentration of 5 per cent.

amounts are measured as any deposits which have formed must be included. The whole is mixed and poured into plates which are dried in the incubator before use. Nos. 5 and 6 keep for months. The final medium should be used on the day it is prepared.

TETRATHIONATE BROTH

(1)

Chalk	25 g.
Broth	900 ml.

The chalk should have been autoclaved and dried before being added to the broth.

Steam for 30 minutes.

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Sodium thiosulphate (crystals)	.	600 g.
Water	.	to 1000 ml.

Steam for 30 minutes.

(3)

Iodine	60 g
Potassium iodide	50 g
Water	to	200 ml.

Grind the iodine and potassium iodide in a mortar before the water is added.

(4)

No. 1	18 ml
No. 2	20 ml.
No. 3	4 ml.

Mix the constituents before measuring and the mixture before dispensing. Dispense, with sterile precautions, in sterile tubes, 5 to 10 ml. in each.

The medium as prepared for use should not be kept for more than a few days. The constituents 1, 2 and 3 keep indefinitely.

Adjust pH to 6·8, dispense in 5 ml. amounts in tubes and autoclave. When cold, add 0·2 ml of 5 per cent bovine albumin (plasma fraction V), sterilised by filtration, and 0·25 ml of a 10 per cent. glucose solution, sterilised by autoclaving. Bacto Caseitone (an enzymatic digest of casein) may be obtained from the Difco Laboratories, Detroit; Tween 80 (a water soluble synthetic ester of oleic acid) from the Atlas Powder Co., Wilmington, Delaware, and bovine plasma fraction V from the Armour Laboratories, Chicago.

Media for the Culture of Anaerobic Bacteria
MINCED MEAT MEDIUM (ROBERTSON)

Minced beef, free from fat	500 g
N/20 NaOH	500 ml

Add the meat to the liquid while the latter is boiling and simmer for 20 minutes. Strain through muslin and discard the fluid. Spread the meat on filter paper to dry. Put the dry meat in tubes or 1 oz screw cap bottles to a depth of about 2 cm and add broth. If only a small amount of broth is used, giving a layer of about 2 cm. in depth, the tubes or bottles should be heated in a water bath at 100° for half an hour to expel air and the surface of the broth covered with a layer of liquid paraffin 1 cm. deep. By using narrow tubes and increasing the depth of the broth layer to 5 cm., the oil may be dispensed with. Sterilise in the autoclave at 115° for 1 hour. Tubes or bottles of this medium should be heated in boiling water for a few minutes to expel dissolved oxygen and cooled immediately before use.

THIOGLYCOLLATE MEDIUM (BREWER)

Sodium thioglycollate	1 g.
Agar powder	0·5 g.
Glucose	5 g.
1 per cent. aqueous methylene blue	0·2 ml.
Broth	1000 ml.

Steam to dissolve the agar. Dispense in tubes to a depth of 8 cm. Autoclave at 115° for 1 hour.

The methylene blue is an indicator of the oxygen-reduction

✓ LOWENSTEIN-JENSEN MEDIUM

Potassium dihydrogen phosphate

(KH₂PO₄) 2.40 g.

Magnesium sulphate 0.24 g.

Magnesium citrate 0.60 g.

Asparagin 3.60 g.

Glycerol 12 g.

Water 600 ml.

Dissolve with the aid of heat and allow to cool. Add:

Potato starch 30 g.

Mix and heat in a water bath at 56° with constant stirring for about 20 minutes by which time a satisfactory paste should be produced. Continue heating at 56° for 1 hour.

A sufficient number (20-22) of fresh eggs are used to yield 100 ml. of egg-yolk mixture. They are prepared, mixed and filtered as for Griffith's medium, but without the addition of saline or basic fuchsin. Mix the salt-starch paste (at 56°) and the egg fluid and add:

2 per cent. aqueous malachite green . . . 20 ml.

Dispense in 5 ml. amounts in 1 oz. screw cap bottles. These are heated in a horizontal position in an inspissator at 75° for half an hour. They are left in the inspissator overnight and again heated at 75° for half an hour.

DUBOS MEDIUM

Potassium dihydrogen phosphate

(KH₂PO₄) 1.0 g.

Disodium hydrogen phosphate

(Na₂HPO₄ · 12H₂O) 6.5 g.

Calcium Chloride 0.0005 g.

Magnesium sulphate 0.001 g.

Zinc sulphate 0.0001 g.

Copper sulphate 0.0001 g.

Ferric ammonium citrate 0.05 g.

Asparagine 2.0 g.

Bacto caseitone 2.0 g.

Tween 80 0.5 g.

Water 1000 ml.

Adjust pH to 6·8, dispense in 5 ml amounts in tubes and autoclave. When cold, add 0·2 ml. of 5 per cent bovine albumin (plasma fraction V), sterilised by filtration, and 0·25 ml. of a 10 per cent. glucose solution, sterilised by autoclaving. Bacto Casitone (an enzymatic digest of casein) may be obtained from the Difco Laboratories, Detroit, Tween 80 (a water soluble synthetic ester of oleic acid) from the Atlas Powder Co., Wilmington, Delaware; and bovine plasma fraction V from the Armour Laboratories, Chicago.

Media for the Culture of Anaerobic Bacteria
MINCED MEAT MEDIUM (ROBERTSON)

Minced beef, free from fat	500 g.
N/20 NaOH	500 ml

Add the meat to the liquid while the latter is boiling and simmer for 20 minutes. Strain through muslin and discard the fluid. Spread the meat on filter paper to dry. Put the dry meat in tubes or 1 oz. screw cap bottles to a depth of about 2 cm. and add broth. If only a small amount of broth is used, giving a layer of about 2 cm. in depth, the tubes or bottles should be heated in a water bath at 100° for half an hour to expel air and the surface of the broth covered with a layer of liquid paraffin 1 cm deep. By using narrow tubes and increasing the depth of the broth layer to 5 cm, the oil may be dispensed with. Sterilise in the autoclave at 115° for 1 hour. Tubes or bottles of this medium should be heated in boiling water for a few minutes to expel dissolved oxygen and cooled immediately before use.

THIOGLYCOLLATE MEDIUM (BREWER)

Sodium thioglycollate	1 g.
Agar powder	0·5 g.
Glucose	5 g.
1 per cent. aqueous methylene blue	0·2 ml.
Broth	1000 ml.

Steam to dissolve the agar. Dispense in tubes to a depth of 8 cm. Autoclave at 115° for 1 hour.

The methylene blue is an indicator of the oxygen-reduction

potential. If more than a very narrow zone at the surface is of a blue colour, the tubes should be immersed in boiling water and cooled before use.

Media for Differentiating Coliform Bacteria

BUFFERED GLUCOSE BROTH

Peptone (preferably Difco proteose)	5 g.
Glucose	5 g.
Dipotassium hydrogen phosphate (K_2HPO_4)	5 g.
Water	800 ml.

Steam to dissolve the peptone. Cool. Filter. Make up volume to 1000 ml. Dispense in tubes, 10 ml. in each. Sterilise in steamer by the intermittent method.

✓ CITRATE MEDIUM (KOSER)

Sodium ammonium phosphate .	1.5 g.
Potassium dihydrogen phosphate	1 g.
Magnesium sulphate	0.2 g.
Sodium citrate (crystals) . .	2.5 g.
Water	1000 ml.

Dispense in tubes, 10 ml. in each. Sterilise by autoclaving at 115° for 15 minutes.

Handwritten: Probability Table
MCCRADY'S PROBABILITY TABLE

Number of samples and volume of each.			Probable number of <i>Bact. coli</i> per 100 ml of water
1× 50 ml	5× 10 ml.	5× 1 ml.	
Number giving positive result			
0	0	0	0
0	0	1	1
0	0	2	2
0	1	0	1
0	1	1	2
0	1	2	3
0	2	0	2
0	2	1	3
0	2	2	4
0	3	0	3
0	3	1	5
0	4	0	5
1	0	0	1
1	0	1	3
1	0	2	4
1	0	3	6
1	1	0	3
1	1	1	5
1	1	2	7
1	1	3	9
1	2	0	5
1	2	1	7
1	2	2	10
1	2	3	12
1	3	0	8
1	3	1	11
1	3	2	14
1	3	3	18
1	3	4	20
1	4	0	13
1	4	1	17
1	4	2	20
1	4	3	30
1	4	4	35
1	4	5	40
1	5	0	25
1	5	1	35
1	5	2	50
1	5	3	90
1	5	4	160
1	5	5	180+

Modified from *The Bacteriological Examination of Water Supplies*,
 Report No 71, Ministry of Health, London, 1939

potential. If more than a very narrow zone at the surface is of a blue colour, the tubes should be immersed in boiling water and cooled before use.

Media for Differentiating Coliform Bacteria

BUFFERED GLUCOSE BROTH

Peptone (preferably Difco proteose)	5 g.
Glucose	5 g.
Dipotassium hydrogen phosphate (K_2HPO_4)	5 g.
Water	800 ml.

Steam to dissolve the peptone. Cool. Filter. Make up volume to 1000 ml. Dispense in tubes, 10 ml. in each. Sterilise in steamer by the intermittent method.

✓ CITRATE MEDIUM (KOSER)

Sodium ammonium phosphate .	1.5 g.
Potassium dihydrogen phosphate	1 g.
Magnesium sulphate . . .	0.2 g.
Sodium citrate (crystals) . . .	2.5 g.
Water	1000 ml.

Dispense in tubes, 10 ml. in each. Sterilise by autoclaving at 115° for 15 minutes.

Anitoxin—*cont*

- Tetanus, 428, 429
- Antitoxins, Toxins and, 219–225
- Arthus reaction, 245, 246
- Aseptic surgery, 155
- Atebrin, 498
- Atopy, 243, 248, 249
- Attenuation of bacteria, 203, 204
- Aureomycin, 163, 164
- Autoclave, 40, 41
- Auto-intoxication, 187
- Autotrophic bacteria, 6, 185
- Avenue of infection, 194
- Avidity of antitoxin, 220
- A P S, 90
- A P T, 312

B

- Bacillaceæ, 251
- Bacille Calmette-Guérin, 396, 397
- Bacilli, 10, 11, 12
- Bacillus, 251, 254
 - anthracis*, 139, 142, 293–300
 - brevis*, 163
 - mesentericus*, 300
 - subtilis*, 300, 301
- Bacteræmia, 197, 198
- Bacteria, Classification of, 250, 255
 - Enumeration of, 82–86
 - General properties of, 1–5
 - in health and disease, 185–198
 - Killing, by heat, 37–41
 - Microscopic observation of, 15–36
 - Nutrition of, 5–9
- Bacteriaceæ, 251
- Bactericidal effect of antibodies, 213
 - substances, 149
- Bactericidin, 214, 238
- Bacterioidæ, 251
- Bacteriolysin, 214, 239
- Bacteriolytic effect of antibodies, 213
- Bacteriophage, 484–486
 - Typing of *Salmonella typhi* by, 96–101, 146
- Bacteriostatic substances, 149
- Bacteriotropin, 217
- Bacterium, 10
- Bacterium, 251, 253, 317
 - aerogenes*, 56, 61, 176, 321, 322
 - cloacæ*, 322
 - coli*, 53, 56, 57, 61, 139, 142, 176–178, 317–320
 - pneumoniae*, 139, 322, 324
- Bagdad button, 509
- Bang, 380
- Barritt's method, 143, 144
- Besredka, 214
- Bed bug, 410
- Behring venule, 135
- Benign lymphocytic chorio-meningitis, 468, 469
- Bergey's Manual, 250
- Berkefeld filter, 87–89
- Bismarck brown, 27, 521
- Biotin, 8
- Blastomycetes, 518
- Blepharoplast, 502
- Blood agar, 70, 71, 140
 - Citrated, 62
 - culture, 131–134, 341
 - film, Preparation of, 21, 22
 - for culture media, 62
 - Obtaining, from animals, 90, 91
- Boas-Oppler bacillus, 358
- Boeck and Drbohlav, Medium of, 502
- Boiled blood agar, 50
- Boils, 259
- Bordet, 238
 - and Gengou, 240
 - Medium of, 377
 - Gengou phenomenon, 241
- Borrelia, 251, 254, 408
 - berbera*, 410
 - carteri*, 410
 - duttonii*, 410
 - novyi*, 410
 - obermeieri*, 408–410
 - vincentii*, 421
- Boström, 448
- Botulism, 430–434
- Bottles, Screw-cap, 49
- Brill's disease, 488
- Brazilian spotted fever, 490
- Brewer's medium, 61, 533
- Broth, 43, 49
 - Buffered glucose, 61, 143, 144, 534
 - Lemco, 524
 - Tetrahionate, 58
 - Wright's, 524
- Brownian movement, 3, 20
- Bruce, 380
- Brucella, 251, 253
 - abortus*, 9, 134, 182, 380–383
 - melitensis*, 380–382
 - suis*, 380, 381, 383
- Brucellæ, 251
- Bubo, 480
- Buffered glucose broth, 61, 143, 144, 534

INDEX

A

- Abortion in cows, 382, 383
 Absorption of agglutinin, 110, 111, 145, 229
 Acetic acid, 7
 Acetyl-methyl-carbinol, 7, 143
Achorion quinceanum, 516
schoenleinii, 516
 Achromobacteriaceæ, 251
 Acid alcohol, 520
 Acid- and alcohol-fastness, 384, 385
 Acne, 316
 Acridines, 156
Actinobacillus, 251, 253, 447
actinomycetemcomitans, 451
lignieresii, 451
Actinomyces, 137, 251, 254
bovis, 447-451
griseus, 163
israeli, 450
lavandulæ, 164
maduræ, 450
muris, 423
 Actinomycetaceæ, 251
 Actinomycetales, 251
 Actinomycosis, 447-451
 Adhesion test, 420
Adjuvants, 192
Aedes aegypti, 475, 476
Aerobacter, 251, 252
aerogenes, 321
cloacæ, 322
 Aerobes, 9
 Agar, Nutrient, 45, 47, 50, 525
 slope, 76
 Sloppy, 50
 Agglutination, 213
 Floccular, 228
 Granular, 228
 Macroscopic method of, 105-107
 method, Felix's, 108-110
 Microscopic method of, 105
 Precipitation and, 226-237
 Slide, 103-105
 tests, 102-110, 343-345
 Agglutinin, 214, 227
 Absorption of, 110, 111, 145, 229
 Agranulocytosis, 158
 Aggressiveness, 192
 Albert's stain, 32, 520
Alcaligenes, 251, 252
faecalis, 357
 Alcohol, 7, 153, 154
 Disinfecting power of, 153, 154
 Alexin, 238
 Alimentary tract, Bacteria of, 186, 187
 Alkaline methylene blue stain, 521
 Allergy, 243, 249
 All-glass syringe, 132
 Alum precipitated toxoid, 312
 Amboceptor, 238 *Halmothyric Synter*
 Amœbic dysentery, 499-502
 Anaerobes, 9
 Media for, 60, 61, 533
 Anaerobic bacteria of wounds, 435-446
 cellulitis, 445
 methods, 77-80
 myositis, 445
 Anaphylactogen, 244
 Anaphylaxis, 243-248
 Andrade's indicator, 52, 519
 Andrewes, 232
 Animal inoculation, 90-93
 Animals, Diseases of, 190
 Antagonism, 75
Anthrax, 293-300
 Anti-anaphylaxis, 246
 Antibiotics, 158-174
 Antibody, Definition of, 209
 Antibodies, Antigens and, 208-214
 Chemical nature of, 211
 Antigen, Definition of, 209
 Kahn, 121
 mosaic, 212
 Wassermann, 115, 116
 Antigens, 146
 and antibodies, 208-214
 Flagellar and somatic, 231
 H, O and R, 231, 236
 of salmonella, 327, 330
 of *Salmonella*, 235
 Antitoxin, Diphtheria, 306, 307, 314
 Ehrlich's unit of, 222

Antitoxin—*cont.*

- Tetanus, 428, 429
- Antitoxins, Toxins and, 219-225
- Arthus reaction, 245, 246
- Aseptic surgery, 155
- Atebrin, 498
- Atopy, 243, 248, 249
- Attenuation of bacteria, 203, 204
- Aureomycin, 163, 164
- Autoclave, 40, 41
- Auto-intoxication, 187
- Autotrophic bacteria, 6, 185
- Avenue of infection, 194
- Avidity of antitoxin, 220
- A.P.S., 90
- A.P.T., 312

B

- Bacillaceæ, 251
- Bacille Calmette-Guérin, 396, 397
- Bacilli, 10, 11, 12
- Bacillus*, 251, 254
 - anthracis*, 139, 142, 293-300
 - brevis*, 163
 - mesentericus*, 300
 - subtilis*, 300, 301
- Bacteræmia, 197, 198
- Bacteria, Classification of, 250, 255
 - Enumeration of, 82-86
 - General properties of, 1-5
 - in health and disease, 185-198
 - Killing, by heat, 37-41
 - Microscopic observation of, 15-36
 - Nutrition of, 5-9
- Bacteriaceæ, 251
- Bactericidal effect of antibodies, 213
 - substances, 149
- Bactericidin, 214, 238
- Bacterioidææ, 251
- Bacteriolysin, 214, 239
- Bacteriolytic effect of antibodies, 213
- Bacteriophage, 484-486
 - Typing of *Salmonella typhi* by, 96-101, 146
- Bacteriostatic substances, 149
- Bacteriotropin, 217
- Bacterium, 10
- Bacterium*, 251, 253, 317
 - aerogenes*, 56, 61, 176, 321, 322
 - cloacæ*, 322
 - coli*, 53, 56, 57, 61, 139, 142, 176-178, 317-320
 - pneumoniae*, 139, 322, 324
- Bagdad button, 509

- Bang, 380
- Barritt's method, 143, 144
- Besredka, 214
- Bed bug, 410
- Behring venule, 135
- Benign lymphocytic chorio-meningitis, 468, 469
- Bergey's *Manual*, 250
- Berkefeld filter, 87-89
- Bismarck brown, 27, 521
- Biotin, 8
- Blastomycetes, 518
- Blepharoplast, 502
- Blood agar, 70, 71, 140
 - Citrated, 62
 - culture, 131-134, 341
 - film, Preparation of, 21, 22
- Bordet, 238
 - and Gengou, 240
 - Medium of, 377
 - Gengou phenomenon, 241
- Borrelia*, 251, 254, 408
 - berbera*, 410
 - carieri*, 410
 - duttoni*, 410
 - novyi*, 410
 - obermeieri*, 408-410
 - vincenti*, 421
- Bostrom, 448
- Botulism, 430-434
- Bottles, Screw-cap, 49
- Brill's disease, 488
- Brazilian spotted fever, 490
- Brewer's medium, 61, 533
- Broth, 43, 49
 - Buffered glucose, 61, 143, 144, 534
 - Lemco, 524
 - Tetrathionate, 58
 - Wright's, 524
- Brownian movement, 3, 20
- Bruce, 380
- Brucella*, 251, 253
 - abortus*, 9, 134, 182, 380-383
 - melitensis*, 380-382
 - suis*, 380, 381, 383
- Brucellææ, 251
- Bubo, 480
- Buffered glucose broth, 61, 143, 144, 534

INDEX

A

- Abortion in cows, 382, 383
- Absorption of agglutinin, 110, 111, 145, 229
- Acetic acid, 7
- Acetyl-methyl-carbinol, 7, 143
- Achorion quinckeum*, 516
- schoenleinii*, 516
- Achromobacteriaceæ, 251
- Acid alcohol, 520
- Acid- and alcohol-fastness, 384, 385
- Acne, 316
- Acridines, 156
- Actinobacillus*, 251, 253, 447
- actinomycescomitans*, 451
- lignieresii*, 451
- Actinomyces*, 137, 251, 254
- bovis*, 447-451
- griseus*, 163
- israeli*, 450
- lavandulæ*, 164
- maduræ*, 450
- muris*, 423
- Actinomycetaceæ, 251
- Actinomycetales, 251
- Actinomycosis, 447-451
- Adhesion test, 420
- Adjuvants, 192
- Aedes aegypti*, 475, 476
- Aerobacter*, 251, 252
- aerogenes*, 321
- cloacæ*, 322
- Aerobes, 9
- Agar, Nutrient, 45, 47, 50, 525
- slope, 76
- Sloppy, 50
- Agglutination, 213
- Floccular, 228
- Granular, 228
- Macroscopic method of, 105-107
- method, Felix's, 108-110
- Microscopic method of, 105
- Precipitation and, 226-237
- Slide, 103-105
- tests, 102-110, 343-345
- Agglutinin, 214, 227
- Absorption of, 110, 111, 145, 229
- Agranulocytosis, 158
- Aggressiveness, 192
- Albert's stain, 32, 520
- Alcaligenes*, 251, 252
- faecalis*, 357
- Alcohol, 7, 153, 154
- Disinfecting power of, 153, 154
- Alexin, 238
- Alimentary tract, Bacteria of, 186, 187
- Alkaline methylene blue stain, 521
- Allergy, 243, 249
- All-glass syringe, 132
- Alum precipitated toxoid, 312
- Amboceptor, 238 *Hæmolytic system*
- Amœbic dysentery, 499-502
- Anaerobes, 9
- Media for, 60, 61, 533
- Anaerobic bacteria of wounds, 435-446
- cellulitis, 445
- methods, 77-80
- myositis, 445
- Anaphylactogen, 244
- Anaphylaxis, 243-248
- Andrade's indicator, 52, 519
- Andrewes, 232
- Animal inoculation, 90-93
- Animals, Diseases of, 190
- Antagonism, 75
- Anthrax, 293-300
- Anti-anaphylaxis, 246
- Antibiotics, 158-174
- Antibody, Definition of, 209
- Antibodies, Antigens and, 208-214
- Chemical nature of, 211
- Antigen, Definition of, 209
- Kahn, 121
- mosaic, 212
- Wassermann, 115, 116
- Antigens, 146
- and antibodies, 208-214
- Flagellar and somatic, 231
- H, O and R, 231, 236
- of salmonellæ, 327, 330
- Antigenic formula of *Salmonella*, 235
- Antiseptics, 149-151, 154
- Antisera, Preparation of, 91-93
- Antitoxic serum, Preparation of, 221, 222
- Antitoxin, Diphtheria, 306, 307, 314
- Ehrlich's unit of, 222

Dehydrogenase, 6, 7
 Delhi boil, 509
 Dengue fever, 483
Dermacentor andersoni, 372
 Desoxycholate-citrate medium, 56, 130, 140, 327, 528
 Dettol, 14, 153
 Dick test, 271
 Dick toxin, 267, 270
 Dieudonné's medium, 364
 Differential media, 44
 Diphasic variation, 232-234
 Diphtheria, 125, 126, 302-315
 Diphtheroid bacillus, 315
 Diplococci, 10, 11
Diplococcus, 251, 252
 pneumoniae, 276-282
 Disease, How bacteria produce, 195, 196
 Dishes, Petri, 63
 Disinfectant, 149, 153
 Doderlein's bacillus, 358
 Domagk, 156
 Double staining, 27-31
 Doulton filter, 87-89
 Dreyer's agglutination method, 102, 105-7
 Dubos, Medium of, 59, 60, 386, 387, 532
 Ducrey, 379
 Dum-dum fever, 507
 Duran-Reynals, Spreading factor of, 193
 Durham tube, 52
 Dust, 189, 190
 Dysentery, Amoebic, 499-502
 Bacillary, 347-353
 Dysgonic, 387

E

Eberthella typhosa, 335
 Egg media, 59, 386
 Ehrlich, 156, 222, 238
 and Morgenroth, 240
 Ehrlich's reagent, 510
 Rosindol reaction, 143
 Electron microscope, 16, 17
 Elementary body, 453, 458, 460
 El Tor vibrio, 364
 Embden-Meyerhof-Parnas scheme, 7
 Emetine, 502
 Encephalitis, 468, 469
 Endocarditis, 188, 270
 Endometritis, 291
 Endotoxins, 196, 197

Energy, Sources of bacterial, 6
 Enumeration of bacteria, 82-86
Entamoeba coli, 500, 501
 histolytica, 347, 499-502
 Enteric bacilli in milk, 181
 water, 178
 fever, 332, 335-342
 Enterobacteriaceae, 251
 Enterococcus, 274
 Enterotoxin of *Staph. pyogenes*, 258
 Erysipelas, 270
 Erythrogenic toxin, 267, 271
 Escherichiae, 251
Escherichia, 251, 252
 coli, 318
 Essential growth factor, 8
 Ethyl alcohol, 7
 Eubacteriales, 250
 Eugonic, 387
 Excreter, 188
 Exotoxins, 196, 197

F

Faeces, Bacteria in, 187
 Examination of, 130
 False membrane, 305
 Farcy, 406
 Favus, 516
 Felix, 329, 330, 339, 340
 Agglutination method of, 102, 108-110
 Fermentation, 7, 144
 Fibrinolysin, 268
 Fièvre boutonneuse, 490
 Films, Preparation and staining of, 20-26
 Preparation of blood, 21, 22
 Filter, Various types of, 87-89
 Filterable virus, 454
 Filtration, 46, 47, 87-90, 454
 Fungi, Pathogenic, 511-518
 Fission in bacteria, 3, 4
 Fixation of complement, 241, 242
 Flagella, 2, 3
 Flea, Rat, 369, 370
 Fleming, 155, 159
 Flocculation tests, 242
 Floccular agglutination, 228
 Florey and Chain, 159
 Fluorescence microscopy, 35, 36
 Folic acid, 8
 Fontana's stain, 32, 33, 522
 Food poisoning, *Salmonella*, 333, 334
 Foot and mouth disease, 483

Buffered glycerol saline, 348, 527
 Buffers, Use of, in media, 46
 Butyl alcohol, 7
 Butylene glycol, 7
 Butyric acid, 7
 B C G., 397
 β -alanine, 8

C

- Calf lymph, 472
 Calmette-Guérin, 396
 Canada balsam, 24, 25
 Capillary pipette, 64, 65
 Capsule, Bacterial, 2
 stains, 33, 34
 Carbohydrate media, 51-53
 Carbol fuchsin, 26, 521
 saline, 62, 519
 thionin blue, 27, 521
 Carbolic acid, 154
 co-efficient, 152
 Carbon dioxide, 9, 80, 81
 Carbuncles, 259
 Carrier, 188, 260, 338, 339, 345, 346 ✓
 Catarrhal jaundice, 482
 Cedar oil, 15
 Cellulitis, 269
 Anaerobic, 445
 Centrifuge, 63, 64
 Cerebro-spinal fluid, Examination of,
 128, 129
 Curate, 519
 medium, Koser's, 534
 Curated blood, 62
 Chaga's disease, 506
 Chamberland filter, 87-89
 Chancre, Examination of, 131
 Soft, 379
 Chemical composition of bacteria, 4, 5
 Chemotherapy, 154-158
 Chick embryo, 455
 -Martin test, 153
 Chiniofon, 502
 Chlorine, 153
 Chloromycetin, 163, 164
 Chocolate agar, 50, 374
 Cholera, 360-364
 red reaction, 143, 361
 Chorio-meningitis, Benign lymphocytic,
 468, 469
 Chrysoidin, 521
 Chrysops discalis, 371
 Classification of bacteria, 250-255
 Climatic bubo, 379
 Clostridia, Toxin producing, 424-
 Clostridial cellulitis, 445
 myositis, 445
 Clostridium, 251, 254, 424, 435, 437
 botulinum, 430-434
 histolyticum, 441, 442
 novyi, 441
 oedematiens, 441
 perfringens, 436-440
 septicum, 439-440
 sporogenes, 442, 443
 tetani, 139, 219, 424-430
 welchii, 436
 Coagulase, 258-260
 Coagulated serum, 526
 Cocci, 10, 11
 Co-enzymes, 7, 8
 Cold agglutinins, 480, 481
 Effect of, on bacteria, 9
 Coliform bacteria, 61, 62, 317-324
 Colony, Appearance of, 140, 141
 Comma bacillus, 360
 Common cold, 483
 Complement, 115, 117, 118, 238-242
 Fixation of, 214, 241, 242
 Contagious diseases, 190
 Copper, Effect of, in media, 43, 44
 Corynebacteriaceae, 251
 Corynebacterium, 251, 252
 acnes, 316
 diphtheriae, 26, 31, 32, 53-55, 138,
 139, 145, 302-315, 526, 527
 pseudodiphtheriticum, 309, 315
 xerosis, 316
 Councilman bodies, 476
 Counter-staining, 29
 Cover slip, 15
 Cowpox, 203
 Craigie, 330
 Crystal violet, 26, 522
 Culture media, Preparation and use of,
 43-62
 Cultures, Making, 63-81
 Cup plate method, 168, 169
 Cylin, 153
 Cystitis, 188
 Cytochrome, 7
 Cytolysin, 239
 Cytoplasmic inclusion bodies, 457, 458
 C. S. K. I. C. I. C. I. A.
 D
 Dale's theory of anaphylaxis, 246, 247
 Dark ground illumination, 16

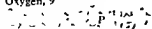
- Immunity—*cont.*
 Passive, 205-207
 Species, 201
 Tissue, 201, 202
 Impetigo, 259
 Incubating plates, 70
 Incubator, 81
 Inclusion body, 457, 458
 Indian ink method, 25, 26, 32
 Indicator organisms, 176
 Indole, 49, 142, 143
 Influenza, 477-479
 Inhibition, Zones of, 228
 Inoculation against smallpox, 203, 471
 Animal, 90-93
 Inositol, 8
 Insect borne disease, 190
 Inspissation, 54
 Intermediate variety of *C. diphtheria*, 304, 305
 Intermittent sterilisation, 39
 Intestinal bacteria, Media for, 55-59, 527-531
 diseases, 190
 Invasion without infection, 191
 Iodine, 153
 Iso-agglutinins, 237
 Izal, 153
- J**
- Japanese encephalitis, 468
 Jaundice, Catarrhal, 482
 Epidemic, 417-420
 Homologous serum, 482
 Jenner, 203, 471, 472
- K**
- Kahn test, 121-124, 415
 Kala azar, 507
 Kenya fever, 490
Klebsiella, 251, 253
pneumoniae, 322
 Koch's postulates, 147, 148
 Koch-Weeks bacillus, 376
 Koser's citrate medium, 61, 62, 319, 322, 323, 534
 Kupffer cells, 215
- L**
- Labels, Gummed, 14
 Laboratory rules, 13, 14
 Lactic acid, 7, 154
Lactobacillæ, 251
Lactobacillus, 251, 252, 357, 358
acidophilus, 358
bifidus, 358
bulgaricus, 358
odontolyticus, 358
Lactobacteriaceæ, 251
 Lactose bile salt broth, 44, 526
 Lancefield's grouping of streptococci, 263, 264
 Lazear, 477
 Leifson's medium, 56, 528
 Leishman-Donovan body, 507
Leishmania donovani, 507, 508
infantum, 508
tropica, 509
 Leishmaniasis, 507-510, 522
 Leishman's stain, 32, 35
 Lepa cells, 403
 Leprosy, 402-404
Leptospira, 251, 254, 408, 417-420
biflexa, 419
canicola, 419
grippe-typhosa, 420
hebdomadis, 420
icterohæmorrhagica, 417-420
 Leucocidin of *Staph. pyogenes*, 258
 Leucocytes, Polymorphonuclear, 201
 Levaditi's method, 33
 Light, Effect of, on bacteria, 10
 for microscope, 15
 Lignieres and Spitz, 451
 Lister, 154
 Löffler, 405
 Löffler's alkaline methylene blue, 26, 521
 medium, 53, 54, 526
 Louse, 410, 488
 Lowenstein-Jensen medium, 59, 532
 Lugol's iodine, 522
 Lysozyme, 200
 Lymphangitis, 269
 Lymphogranuloma venereum, 379, 479, 480
 Lysol, 13, 153
 L D 50, 152, 194
 Lf dose, 223, 307
 Lr dose, 223, 307
 L + dose, 224, 307
- M**
- MacConkey's medium, 53, 55, 56, 327, 342, 527
 McCrady's tables, 86, 176, 535

- Formalin, 154
 Formic acid, 7
 Formol toxoid, 312
 Fractional sterilisation, 39
 Frambæsia, 416
 Francis, 477
 Frei test, 480
 Fuchsin, Carbol, 26
Fusobacterium, 251, 253
 plauti-vincenti, 421
Fusiform bacilli, 421
Fusiformis fusiformis, 421
 F.T., 312
- G**
- Gaffkya*, 250, 252
 tetragena, 261, 262
 Gangrene, Gas, 188, 443-446
 Gardner and Leslie, 378
 Gas gangrene, 188, 443-446
 Gastro-enteritis, 332
 Geissler's vacuum pump, 63
 Gelatin, 45, 47, 51, 525
 Liquefaction of, 142
 Gelatinase, 257
 Genitals, Bacteria of, 187
 Examining material from, 131
 Gentian violet, 153
 media, 44, 51
 German measles, 483
 Germicide, 149
 Gingivitis, Acute ulcerative, 421
 Glanders, 405-407
 Globulin, 211
Glossina morsitans, 506
 palpalis, 504
 Glucose, 520
 Glutamine, 8
 Glycerol, 154
 Gonorrhœa, 288-292
 Gonococcus, 142, 288-292
 Gradocol membranes, 90
 Gram positive and negative bacteria, 138
 Gram's stain, 24, 27-30
 Gramicidin, 163
 Granular agglutination, 228
 Granuloma venereum, 379
 Gravis variety of *C. diphtheria*, 304, 305
 Griffith's egg medium, 59, 531
 typing of streptococci, 264
 Grouping of hæmolytic streptococci, 112, 113
- Guarnieri bodies, 471, 474
 Gutter plate method, 165-167
- H**
- H antigens, 231, 234-236
 Hæmagglutination by virus, 478
Hæmagglutinina, 237
Hæmatin, 8
 Hæmolytic streptococci, 112, 113, 263, 272, 273
 tests, 113, 114
 Hæmolysin, 115
 Hæmolysins of *Str. pyogenes*, 267
 Hæmolysis, 239
Hæmophilæ, 251
 Hæmophilic bacteria, 374-379
Hæmophilus, 251, 253
 ducreyi, 377, 379
 influenza, 139, 374-376, 477
 pertussis, 377, 378
 Hands, Protection of, 14
 Hanging-drop preparation, 18-20
 Hansen, 402
 Hapten, 209
 Heat, Effect of, on bacteria, 9, 10
 Hepatitis, 482, 483
 Herpes, 469, 470, 474
 Heterotrophic bacteria, 185
 Higher bacteria, 12
 Hiss's method, 33, 34
 Hodgkin's disease, 483
 Hofmann's bacillus, 309, 315, 316
 Horgan and Marshall's medium, 309
 Hot air steriliser, 37, 38
 Hoyle's medium, 309, 310
 Hyaluronidase, 193, 258, 259, 268
 Hydrogen acceptor, 7
 ion, 45, 46
 Hydrophobia, 461
 Hynes's modification of Leifson's medium, 528
 Hypersensitivity, 243-249
 to tuberculin, 395
 Hypochlorites as disinfectants, 154
Helicophila Antichorley, 483
- I**
- Identification of pathogenic bacteria, 137-148
 Idiosyncrasy, 243, 248, 249
 Immune body, 115-117, 214, 238
 Immunity, Acquired, 199, 202-207
 Active, 202-205
 Antitoxic, 204, 205
 Innate, 199-202

- Negri body, 462, 463
Neisseria, 250, 252, 283
 catarrhals, 138, 142, 287
 gonorrhæa, 288-292
 intracellularis, 283-287
 meningitidis, 283-287
Neisseriaceæ, 250
 Neisser's stain, 24, 31, 32, 523
 Neufeld reaction, 279, 282
 Neutral red, 27, 521
 Newcastle strain of, *Sh flexneri*, 351
 New growths, 486
 Nicolauer, 424
 Nicotinic acid, 8
 Nigrosin, 25, 26, 32, 34, 523
 Nitroso-indole test, 142
 Noguchi, 477
 Non-hæmolytic streptococci, 263, 273, 274
 Nostrils, Bacteria of, 186
 Novy, 441
 Novy and MacNeill, Medium of, 508
 Number of bacteria required to infect, 193, 194
 Nutrition of bacteria, 5-9

O

 O antigens, 231, 234-236, 327-329
 Obermeier, 408
 Obtaining material from patient, 125-136
Odium, 518
 Oil, Cedar, 15
 -immersion lens, 15
 Polyric, 15
 Oocyst, 494, 495
 Ookinete, 494, 495
 Opportunists, 185
 Opsonic effect of antibody, 213
 index, 217, 218
 Opsonin, 201, 216-218
 Orchitis, 291
 Oriental sore, 509
 Ornithosis, 479
 Osteomyelitis, 259
 Oxford staphylococcus, 163
 Ozæna, 324
 Oxidation, 6, 7
 Oxygen, 9


 Paludrin, 498
 Pamaquin, 498
 Pantothenic acid, 8
 Pappenheim's stain, 35, 523
Para-aminobenzene sulphonamide, 156
Para-aminobenzoic acid, 8, 157, 179
 Paracolon bacteria, 321
 Paratyphoid fever, 332
Parvobacteriaceæ, 251
 Paschen bodies, 471
 Pasteur, 158, 289, 464
 Pasteur and Joubert, 439
 pipette, 64, 65
Pasteurella, 251, 253, —365-373
 pestis, 35, 139, 365-371
 pseudotuberculosis, 371, 400
 septica, 371
 tularensis, 372, 373
Pasteurellæ, 251
 Pasteurisation, 39, 40
 Pasteurised milk, Bacteria in, 182
 Pathogenic bacteria, 185
 Identification of, 137-148
 fungi, 511-518
 Pathogenicity, 145
 Patient, Examining material from, 125-136
 Paul-Bunnell test, 483
 Paul's test, 474
 Penicillin, 51, 160-172
 Penicillinase, 160, 172
Penicillium notatum, 159
 Pentamide, 510
 Peptone water, 49, 525
 Peritonitis, 188
 Persisters, 161
 Pestis major and minor, 367
 Petri dishes, 63
Pfeifferella mallei, 405
 whitmori, 407
 Pfeiffer's bacillus, 374
 pH of media, 45, 46
 Phage, 146, 330, 331, 484
 Phagocytes, 201
 Phagocytosis, 215-218
 Phase variation of salmonellæ, 236
 Phenol, 154
 red, 45, 46
Phlebotomus, 508
 Phosphatase test, 183
 Phosphorylation, 7
 Piper, 2
 Pimeic acid, 8
 Pipettes, Mouth suction of, 14
 Capillary, 64, 65
 Pasteur, 64, 65
 Plague, 365-371

- McIntosh and Fildes anaerobic jar, 78, 80
- McLeod, 304
- Macacus rhesus*, 476
- Macrogamete, 494, 495
- Macrogametocyte, 493, 494
- Macrophage, 215
- Madura foot, 450
- Malachite green, 522
- Malaria, 491-498
- Malignant growths, 486
- pustule, 297
- Mallein, 407
- Malleomyces*, 251, 253
- mallei*, 405-407
- pseudomallei*, 407
- Malta fever, 382
- Manchester strain of *Sh flexneri*, 351
- Mandler filter, 87
- Mantoux test, 394, 395
- Mastitis, Bovine, 272
- Measles, 481
- German, 483
- Media, Differential, 44
- for anaerobes, 60, 61
- coliform bacilli, 61, 62, 534
- intestinal pathogens, 55-59, 527-531
- Myc. tuberculosis*, 59, 532
- pH of, 45, 46
- Selective, 44
- Mediterranean fever, 382
- Medium, Brewer's, 61, 533-534
- Coagulated serum, 53, 54, 526
- Desoxycholate-citrate, 56, 528, 529
- Dubos, 59, 60, 532, 533
- Griffith's egg, 59, 531
- Koser's citrate, 61, 62, 534
- Leifson's, 56, 528-530
- Löffler's, 53, 54, 526
- Lowenstein-Jensen, 59, 532
- MacConkey's, 55, 56, 527
- Reduced iron, 61
- Robertson's, 60, 61, 533
- Selenite, 59, 531
- Wilson and Blair's, 57, 58, 529, 530
- Melioidosis, 407
- Meningitis, 283, 285
- Meningococcus, 283-287
- Mepacrine, 498
- Merozoite, 492, 494
- Merthiolate, 154
- Metachromatic granules, 303
- Metchnikoff, 215, 442
- Methyl red test, 61, 144, 319, 321, 323
- violet, 26, 523
- Methylene blue, Löffler's, 26, 521
- reduction test, 180
- Micrococaceæ, 250
- Micrococcus*, 250, 251
- melitensis*, 38
- pyogenes*, 256
- Microgamete, 494, 495
- Microgametocyte, 493, 494
- Microphage, 215
- Microscope, 15
- Electron, 16, 17
- Reflecting, 16
- Rules for using, 17, 18
- Microscopic examination, 15-36
- Microsporon audouinii*, 512, 513
- Milk, Examination of, 178-183
- medium, 53
- Minimum hæmolytic dose, 240
- lethal dose, 194, 222
- Mirror test, 111, 229, 230
- Mite typhus, 490
- Mitis variety of *C. diphtheria*, 304, 305
- Moller's method, 34, 35, 397
- Molluscum contagiosum, 483
- Manila, 517, 518
- Mononucleosis, Infectious, 483
- Maraxella*, 251, 253
- Moro test, 396
- Motility, 2, 3, 20
- Mouth, Bacteria of, 186
- Murine typhus, 489
- Mumps, 483
- Mycobacteriaceæ, 251
- Mycobacterium*, 251, 254, 384
- avium*, 384, 387
- butyri*, 397
- lepræ*, 138, 402-404
- muris*, 397
- paratuberculosis*, 397
- smegmatis*, 397, 398
- tuberculosis*, 59, 60, 138, 181, 182, 384-401
- Myonecrosis, 445
- M H D, 240
- M L D, 222, 307
- M R D, 307
- N
- Nagana, 506
- Nagler reaction, 437
- Naphthol, α -, 143, 144

Salmonella—cont

- enteritidis*, 231, 328, 329, 333, 335
- hirschfeldii*, 335
- paratyphi A*, 326–329, 331, 335–346
 - B*, 57, 328, 329, 331, 335–346
 - C*, 331, 333
- schoftmuelleri*, 335
- typhi*, 57, 96–101, 139, 142, 143, 145–147, 229–232, 335–346
- typhimurium*, 232–234, 328, 331, 334, 337
- typhosa*, 335
- Salt agar, 365
- Salvarsan, 156
- Sandfly, 508
- Saprophytic bacteria, 185
- Sarcina*, 10, 11, 250, 252
- Scarlet fever, 270
- Schuck test, 308, 313
- Schizogony, 492
- Schizomycetes, 250
- Schizont, 492, 494
- Sclavo's serum, 300
- Screw cap bottles, 49
- Scrub typhus, 490
- Sections, Staining, 24, 25
- Seitz filter, 46, 47, 89
- Selective media, 44, 54, 55
- Selenite medium, 59, 342, 531
- Sensitised cell suspension, 117
- Sensitivity to penicillin, Tests of, 165–168
- Septicæmia, 198, 269, 270, 333, 334
- Serological identification, 144, 145
 - technique, 102–124
- Serum medium, Coagulated, 53, 54
 - Collection of, 134, 135
 - Sensitivity to, 248
 - sickness, 247
- Seven day fever, 420
- Shedder, 188
- Shellfish, Bacteriology of, 183, 184
- Shigella*, 251, 253, 349–353
 - ambigua*, 350
 - boydii*, 351, 352
 - dysenteriae*, 350
 - (Boyd), 351, 352
 - flexneri*, 139, 140, 350, 351
 - paradysenteriae*, 350, 351
 - schmitzii*, 350
 - shigae*, 350
 - sonnei*, 56, 352, 353
- Sintered glass filter, 88, 89
- Skin, Bacteria of, 185

Skin—cont.

- Disinfection of, 153
 - test dose of Dick toxin, 271
- Slants, agar, 48
- Slide agglutination, 102–105
- Slopes, Agar, 48, 67, 76, 77
- Sloppy agar, 50
- Smallpox, 203, 470
- Smegma bacillus*, 397, 398
- Smith, Andrewes and Laidlaw, 477
- Smith and Brown's classification of streptococci, 263
- Sneeze, 189
- Solutions, Sterile, 62
- Sour milk therapy, 187
- South African tick fever, 490
- Specific soluble substance, 278, 279
- Spirilla, 10, 12
- Spirillæ, 250
- Spirillum*, 250, 251
 - minus*, 422
- Spirochæta*, 408
- Spirochætes*, 10, 12, 32, 33, 408–423
- Spironema vincentii*, 32
- Spirochætales*, 251, 408
- Splenic fever, 296
- Spore stains, 34, 35
- Spores, 4, 10
- Sporoblast, 494, 495
- Sporotrichosis, 516
- Sporotrichum beurmonni*, 516, 517
 - schenkii*, 516
- Sporozoite, 492, 494
- Spreading plate, 71–74
- Spreader, 65, 66
- Sputum, Examination of, 127
- Stabs, Agar, 48
 - Inoculating, 67
- Staining methods, 23–36
- Stains, 520–524
- Staphylococcus*, 10, 11, 142, 256–262
 - albus*, 261
 - citreus*, 261
 - epidermidis*, 261
 - pyogenes*, 147, 256–261
- Steamer, 38, 39
- Stegomyia fasciata*, 475
- Sterile solutions, 62
- Sterilisation, 37–42
- St Louis encephalitis, 468
- Stokes, 476, 477
- Stormy fermentation of milk, 437
- Strangles, 273
- Street virus, 464

- Plasmodium falciparum*, 491
malaria, 491
ovale, 491
vivax, 491, 496
 Plasmogon, 498
 Plate, Spreading, 71-74
 Plates, 63
 Plating, 67-69
 Platinum wire, 63, 64, 67
 Pneumococcus, 276-282
 Pneumonia, 279, 323, 480, 481
 Poliomyelitis, 466-468
 Polychromatic methylene blue, 26
 Polymorphonuclear leucocytes, 215-218
 Polyric oil, 15
 Postulates, Koch's, 147, 148
 Precipitation and agglutination, 226-237
 reactions, Use of, 227
 Precipitin, 214, 226
 Precipitinogen, 226
 Prescott and Breed, Method of, 83, 180, 181
 Primary atypical pneumonia, 480
 Prontosil, 156
 Propyl alcohol, 7
 Propylene glycol as air disinfectant, 154
 Proline, 251
Proteus, 56, 57, 251, 253, 355-357
 mirabilis, 356
 morganii, 356
 vulgaris, 142, 356
 A. K. A. K., 356, 357, 488-490
 Protozoa in blood, 139
 Pathogenic, 491-510
 Psittacosis, 479
Pseudomonadaceae, 250
Pseudomonadaceae, 250
Pseudomonas, 250, 251
 aeruginosa, 354, 355
 pyocyanea, 137, 354, 355
 Puerperal fever, 270
 Pugh's stain, 32, 524
 Pure culture, 44, 45, 71-75
 Purines, 8
 Pus, Collection and examination of, 126, 127
 Pustules, 259
 Pyæmia, 198, 259
 Pyelitis, 188
 Pyocyanase, 158, 355
Pyothroa alveolaris, 421
Pyosalpinx, 291
 Pyridine nucleotides, 7
 Pyridoxine, 8
 Pyrimidines, 8
 Pyruvic acid, 7
 P.P.D., 394, 395
- Q
- Q fever, 487, 490
Quinine, 156, 498
- R
- R antigens, 231
 Rabies, 461-466
 Rabinowitsch, Butter bacillus of, 397
 Ramon, 223
 Rat-bite fever, 422, 423
 Rat flea, 369, 370
 Ray fungus, 447
 Reaction of media, 45, 46
 Reduced iron medium, 61
 Reflecting microscope, 16
 Relapsing fevers, 408-411
 Respiration, 7
 Respiratory tract, Spread of infection from, 189
 Reticulo-endothelial system, 211
Rhinoscleroma, 324
 Riboflavine, 8
Rickettsia, 487-490
 burnetii, 487, 490
 mooseri, 487, 489, 490
 orientalis, 487, 490
 proteaceae, 487, 488, 490
 quintana, 487, 490
 rickettsii, 487, 490
Rickettsiaceae, 251
Rickettsiales, 251
 Rideal-Walker test, 152
 Ringworm, 512
 Robertson's medium, 60, 61, 533
 Rocky mountain spotted fever, 490
 Rosindol, Ehrlich's, reaction, 143
 Ross, 497
 Rough colonies, 141
 Rous sarcoma, 486
 Rules for using microscope, 17, 18
- S
- Safety in the laboratory, 13, 14
 Saline, 62, 520
Salmonella, 110, 111, 231, 234, 251, 253, 325-334
 abony, 329
 choleraesuis, 333

Salmonella—cont

- enteritidis*, 231, 328, 329, 333, 335
 - hirschfeldii*, 335
 - paratyphi A*, 326-329, 331, 335-346
 - B*, 57, 328, 329, 331, 335-346
 - C*, 331, 333
 - schottmuelleri*, 335
 - typhi*, 57, 96-101, 139, 142, 143, 145-147, 229-232, 335-346
 - typhimurium*, 232-234, 328, 331, 334, 337
 - typhosa*, 335
- Salt agar, 365
- Salvarsan, 156
- Sandfly, 508
- Saprophytic bacteria, 185
- Sarcina*, 10, 11, 250, 252
- Scarlet fever, 270
- Schick test, 308, 313
- Schizogony, 492
- Schizomycetes, 250
- Schizont, 492, 494
- Sclavo's serum, 300
- Screw cap bottles, 49
- Scrub typhus, 490
- Sections, Staining, 24, 25
- Seitz filter, 46, 47, 89
- Selective media, 44, 54, 55
- Selenite medium, 59, 342, 531
- Sensitised cell suspension, 117
- Sensitivity to penicillin, Tests of, 165-168
- Septicæmia, 198, 269, 270, 333, 334
- Serological identification, 144, 145
- technique, 102-124
- Serum medium, Coagulated, 53, 54
- Collection of, 134, 135
- Sensitivity to, 248
- sickness, 247
- Seven day fever, 420
- Shedder, 188
- Shellfish, Bacteriology of, 183, 184
- Shigella*, 251, 253, 349-353
- ambigua*, 350
 - boydii*, 351, 352
 - dysenteriae*, 350
 - (Boyd), 351, 352
 - flexneri*, 139, 140, 350, 351
 - paradisenteriae*, 350, 351
 - schmitzii*, 350
 - shiga*, 350
 - sonnei*, 56, 352, 353
- Sintered glass filter, 88, 89
- Skin, Bacteria of, 185

Skin—cont

- Disinfection of, 153
 - test dose of Dick toxin, 271
- Slants, agar, 48
- Slide agglutination, 102-105
- Slopes, Agar, 48, 67, 76, 77
- Sloppy agar, 50
- Smallpox, 203, 470
- Smegma bacillus, 397, 398
- Smith, Andrewes and Laidlaw, 477
- Smith and Brown's classification of streptococci, 263
- Sneeze, 189
- Solutions, Sterile, 62
- Sour milk therapy, 187
- South African tick fever, 490
- Specific soluble substance, 278, 279
- Spirilla, 10, 12
- Spirillæ, 250
- Spirillum*, 250, 251
- minus*, 422
- Spirochaeta*, 408
- Spirochaetes, 10, 12, 32, 33, 408-423
- Spirochaeta vincentii*, 32
- Spirochaetales, 251, 408
- Splenic fever, 296
- Spore stains, 34, 35
- Spores, 4, 10
- Sporoblast, 494, 495
- Sporotrichosis, 516
- Sporotrichum beurmanni*, 516, 517
- schenkii*, 516
- Sporozoite, 492, 494
- Spreading plate, 71-74
- Spreader, 65, 66
- Sputum, Examination of, 127
- Stabs, Agar, 48
- Inoculating, 67
- Staining methods, 21-36
- Stains, 520-524
- Staphylococcus*, 10, 11, 142, 256-262
- albus*, 261
 - citreus*, 261
 - epidermidis*, 261
 - pyogenes*, 147, 256-261
- Steamer, 38, 39
- Stegomyia funiculata*, 479
- Sterile solutions, 62
- Sterilisation, 37-42
- St. Louis encephalitis, 468
- Stokes, 476, 477
- Stormy fermentation of milk, 417
- Strangles, 273
- Street virus, 464

Streptobacillus moniliformis, 423
Streptococcus, 251
Streptococcus, 10, 11, 112, 113, 126,
 142, 181, 251, 252, 263-275
agalactiae, 272, 273
equi, 273
faecalis, 273, 274
mitis, 273, 274
pyogenes, 265-272
viridans, 263

Streptococci, Non-haemolytic, 273, 274

Streptomycin, 163, 173

Streptomyces, 251, 254

Streptothricin, 163

Sub-culture, Making a, 65-67

Substance sensibilisatrice, 238

Succinic acid, 7

Sugar media, 51-53, 525

Sulphanilamide, 156

Sulphonamides, 156-158, 173, 174

Suramin, 505, 506

Susceptibility, 195

Swabs, 125, 126

Swamp fever, 420

Symbiosis, 75

Synthetic medium, 8

Syphilis, 411-416

Syringe, 132, 134

S.S.S., 278, 279

S.T.D., 271,

. T

Technique, Serological, 102-124

Special, 82-101

Tellurite media, 44, 51, 54, 55, 309

Temperature of growth, 9, 142

Tests, Agglutination, 102-110

Tetanus, 424-430

Tetrads, 10, 11

Tetramethyl-*p*-phenylene diamine, 290

Tetrathionate broth, 58, 130, 342, 530

Thiamine, 8

Thermophilic bacteria, 9

Thioglycollate medium, 533

Thionine blue, Carbol, 27

Throat swab, 125, 126

Tick fever, 410, 411

Tissue immunity, 214

Tissues, Examination of, 135, 136

Titre of agglutinating serum, 102, 228

Toxigenicity, 192

Toxins, 196, 197, 219-225

Toxoid, 204, 205, 221, 429, 430

Toxoid-antitoxin floccules, 312

Traum, 380

Tréfouel, 156

Triatoma megista, 506

Trichophyton, 514, 515

Trench fever, 490

Treponema, 251, 254, 408

pallidum, 32, 411, 416

pertenae, 416

recurrentis, 408

vincenti, 421

Treponemataceae, 251

Trophozoite, 492, 494

Tropical ulcer, 421

Trypanosoma brucei, 505, 506

congolense, 506

cruzi, 506

evansi, 503, 506

gambiense, 503, 504

lewisi, 505

rhodesiense, 505

vivax, 506

Trypanosomes, 502-506

Tryparsamide, 505

Tse-tse fly, 504

Tsutsugamushi fever, 490

Tubercle bacillus, 384-401, 531-533

Tubercle, Structure of, 389

Tuberculin, 394-396

Tuberculosis, 384-401

Tubing media, 47, 48

Tularaemia, 372, 373

Types, Differentiation of, 146, 147

Typhoid fever, 230, 332, 335-346

Typhus fever, 487-490

Tyrocidin, 163

T.A.B. (C), vaccine, 329, 330

T.A.F., 312

U

Ultrafiltration, 89, 90

Ultramicroscopic particles, 16

Ultrasonic waves, 10

Undulant fever, 380-383

Undulating membrane, 502

Unit, International, of penicillin, 163

Urine, Examination of, 129, 130

V

V factor, 374, 375

Vaccination, 471, 472

Vaccines, 93-96, 203, 459

Vaccinia, 203, 468, 470

Van Ermengem, 430

Variation, Diphasic, 232-234

aricella, 474
 ariola, 470-474
 enule, 135
 i agglutination, 344-346
 antigen, 231, 329
ibrio, 250, 251
ibrio cholerae, 138, 142, 143, 360-364
 comma, 360
ibrio septique, 439
ibrios, 10, 11
 incant's angina, 126, 421
 iolet, Crystal and methyl, 26
iridans streptococci, 263
ivulence, 192, 193
irus fixe, 464
iruses, 13, 452-487
itamin C deficiency, 200
itamins, 8
 oges-Proskauer reaction, 61, 143, 319,
 322, 323
 oles, 397
 ollmer test, 396
 ulvo-vaginitis, 291

W

-Wart, 483
 Warthin, 413
 Wassermann reaction, 115-121, 242,
 414, 415
 Water, Examination of, 175-178

Water bath, 81
 Weil-Felix reaction, 488
 Weil's disease, 417-420
 Weinberg and Sequin, 441
 Welch and Nuttall, 436
 Wells, 397
 Widal test, 105, 106, 134, 343-345
 Wilson and Blair's medium, 57, 58,
 130, 140, 327, 342, 529
 Wire, Platinum, 63
 Woolsorter's disease, 297
 Wounds, Anaerobic bacteria of, 435-
 446
 Wright, 216
 Wright's broth, 43, 44, 524

X

X factor, 374, 375
 Xerosis bacillus, 316

Y

Yaws, 416
 Yellow fever, 474-477
 Young, 477

Z

Ziehl-Neelsen stain, 24, 30, 31, 138,
 385
 Zones of inhibition, 228
 Zygote, 494, 495

